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Permeability Studies on the Red Blood Cell of the Ground-Hog, *Marmota monax*[1]

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The high degree of permeability exhibited by red blood cells of the ground-hog, *Marmota monax*, to various sugars and polyhydric alcohols has been demonstrated by Parpart ('36) with the hemolysis method. These red cells are more permeable to sugars and polyhydric alcohols than human red cells which are not permeable to the 6-carbon polyhydric alcohols, D-Mannitol and D-Sorbitol. Since Faust ('60) has shown with the photoelectric method on human red cells that variations in pH and temperature greatly affect the rates of penetration of these substances, it had occurred to us to investigate this phenomenon with ground-hog red cells. If the rates of penetration of sugars and polyhydric alcohols into ground-hog red cells are affected in a manner similar to that of human red cells, then this would indicate that perhaps their mechanism of penetration is similar.

METHODS

Approximately 5 ml of blood was obtained by heart puncture from an ether-anesthetized ground-hog. This blood was then quickly placed into a flask containing 40 ml of 1% NaCl-PO_4 at pH 7. No anticoagulants were used. The blood suspension was then washed three times in 1% NaCl-PO_4 at pH 7. Aliquots of this suspension, which was placed in the refrigerator, were prepared for experimentation by the method previously described by Faust ('60). Although Bang and Ørskov ('31), Meldahl and Ørskov ('40), Mawe ('56) and Faust ('60) have observed that glucose penetrates into human red cells more slowly when the blood is not fresh, this did not seem to be the case with ground-hog red cells. There was no noticeable change in the permeability of these cells to sugars and polyhydric alcohols even after they had been stored in the refrigerator for two days. However, washed ground-hog red cells that were more than two days old were never used for experiments.

The relative rates of penetration of various monosaccharides and polyhydric alcohols into ground-hog red cells were studied by means of a photoelectric densimeter, Parpart ('35). Isosmotic concentrations (0.3 M) of these compounds were prepared in an isotonic NaCl-phosphate buffer which was adjusted to a desired pH and then allowed to mutarotate. Exactly 40 mm^3 of a washed red cell suspension was added to 6 ml of the substrate solution which was in the densimeter.

RESULTS

Figure 1 illustrates the effect of pH at 37°C on the rates of penetration to a diffusion equilibrium of some sugars and polyhydric alcohols into ground-hog red cells. The influence of pH on the rates of penetration of the monosaccharides and the polyhydric alcohols is similar. It is obvious that these compounds penetrate more slowly as the pH of their medium becomes more acid. This pH effect is reversible. Incidentally, a similar pH effect was observed on the extremely slow (approximately 40 times slower than D-glucose) rate of penetration of the amino acid, glycine.

Table 1 shows the temperature coefficients (Q_{10}) which were calculated from the rates of penetration of various sugars and polyhydric alcohols at 37°C and 27°C and at pH 7. The temperature coefficients

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[1] This work was done at the Marine Biological Laboratory, Woods Hole, Mass. and supported by the Whitehall Foundation.

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for the sugars ranged between 2.0 and 2.8, whereas those for the polyhydric alcohols were between 1.2 and 1.6. Also from table 1 it can be seen that the rates of penetration of the polyhydric alcohols are approximately related in a linear manner to the number of carbon atoms in their molecule through 5 carbons. This is not true for the various sugars that have been tested.

Neither the disaccharide sucrose nor the cyclic 6 carbon polyhydric alcohol i-inositol were observed to penetrate into the ground-hog red cell. Also, there was no appreciable difference in the rates of penetration of the α- and β-isomers of D-glucose at pH 7 and 37°C.

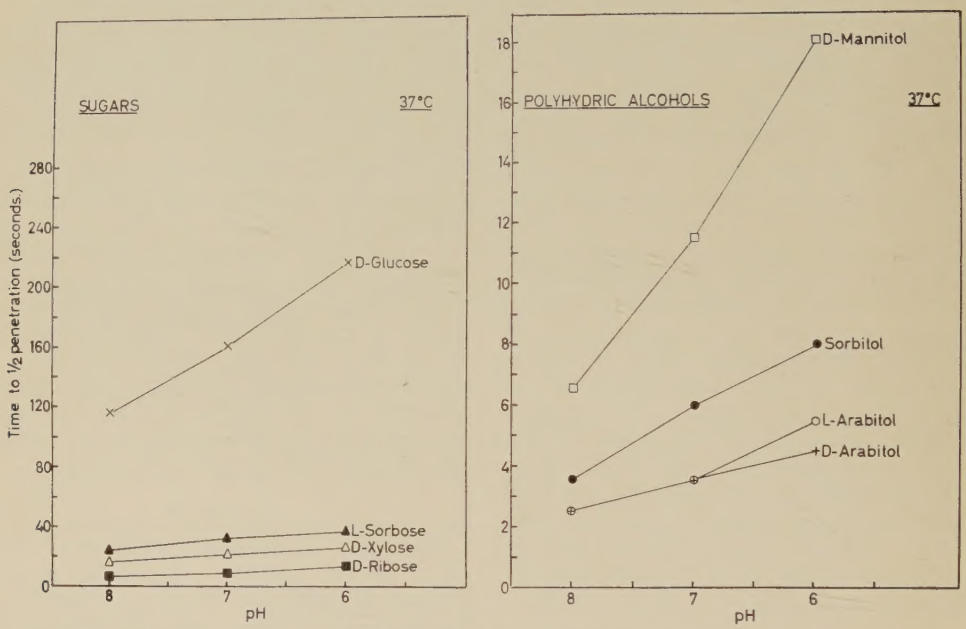

Fig. 1 Effect of pH on the relative rates of penetration of various 0.3 M sugars and polyhydric alcohols in 1% NaCl-PO_4. (Points represent an average of at least three determinations.)

TABLE 1

Results of Q_{10} studies on sugar and polyhydric alcohol penetration at pH 7

(0.3 M of substrate was dissolved in 1% NaCl-PO_4; sugars and alcohols are in order of decreasing penetration)

Sugar	No. of carbon atoms	½ Time penetration at 37°C	½ Time penetration at 27°C	Q_{10}	Polyhydric alcohol	No. of carbon atoms	½ Time penetration at 37°C	½ Time penetration at 27°C	Q_{10}
		seconds	*seconds*				*seconds*	*seconds*	
D-Ribose	5	9.0	21.0	2.3	Glycerol	3	1.0	1.5	1.5
D-Lyxose	5	16.0	36.0	2.2	i-Erythritol	4	2.1	3.0	1.4
D-Xylose	5	22.0	52.0	2.4	D-Arabitol	5	3.5	4.5	1.3
L-Xylose	5	24.0	48.0	2.0	L-Arabitol	5	3.5	4.5	1.3
D-Arabinose	5	24.0	52.0	2.2	D-Xylitol	5	3.5	4.5	1.3
L-Sorbose	6	33.0	72.0	2.2	Ribitol	5	4.0	5.0	1.3
D-Galactose	6	57.0	148.0	2.6	Sorbitol	6	6.0	8.0	1.3
D-Fructose	6	125.0	300.0	2.4	Galactitol	6	9.0	[1]	
D-Glucose	6	160.0	450.0	2.8	D-Mannitol	6	11.5	16.0	1.4

[1] Galactitol was not sufficiently soluble at this temperature and could not be tested.

CONCLUSIONS

The effect of pH on the rates of penetration of 0.3 M sugars and polyhydric alcohols into ground-hog red cells is not the same as that observed by Faust ('60) with human red cells. These compounds penetrate into ground-hog red cells more rapidly as the pH of their medium becomes more alkaline. Whereas in human red cells, D- and L-arabitol and various monosaccharides penetrate more rapidly at pH 7, but, at pH 6 and 8, their rates of penetration are considerably reduced. Also, the effect of pH on the rates of penetration of these compounds is much greater in human red cells than in ground-hog red cells.

The temperature coefficients calculated for sugar penetration into ground-hog red cells is similar to that observed by Faust ('60) with human red cells. However, there exists only a small, if any, relationship between the structural and stereochemical differences among the monosaccharides and their relative rates of penetration into the ground-hog red cell.

These data may indicate that sugars and polyhydric alcohols penetrate into the ground-hog red cell by free diffusion. However, the free diffusion of certain large penetrating molecules may be affected by an interaction between the molecule and the channel in the cell membrane through which it is passing.

ACKNOWLEDGMENT

The authors wish to thank Mr. Earl Van Norman for supplying the ground-hogs used in these experiments.

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The Endogenous Tissue Respiration of the Arctic Ground Squirrel as Affected by Hibernation and Season[1]

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The Arctic ground squirrel, a hibernating species that normally spends upwards of 8 months of the year in an inactive or hibernating state, undergoes marked seasonal changes in body size and metabolism. Thus, after emerging from the burrow in late spring, and for the next month or so thereafter, he is at his minimum weight. Following a period of somewhat slow weight gain during the middle summer months, he quite suddenly starts to put on weight in the form of fat during the first few weeks of fall. Thus, sudden fattening brings him up to his maximum weight for the year. It just precedes the entrance into the burrow for the inactive portion of his yearly life cycle and it is here that he again loses weight in a series of hibernation and arousal periods.

Throughout the yearly cycle, these animals undoubtedly undergo marked shifts in the patterns of tissue metabolism. For instance, in the fall, just prior to hibernation, large amounts of fat are being deposited. Consequently, there must be a favoring of those pathways of metabolism leading from dietary carbohydrate and protein, as well as lipids, toward lipid synthesis. Conversely, during the winter months when the animals are losing weight, pathways of lipid catabolism must be favored. In addition, during the winter periods of alternating hibernation and arousal a number of peculiar, but for the most part unknown, processes of intermediary metabolism probably exist.

These obvious conclusions, however, are based almost entirely on deductive evidence. Direct investigations in the form of tissue respiratory measurements or enzyme activity assays are very limited in number. In fact, as far as could be determined, kidney is the only tissue that has been studied with respect to its seasonal variations in metabolism (Kayser, Jacob and Lucot, '54).

It was felt, therefore, that a survey of the possible seasonal variations in the endogenous respiration of several tissues would be highly desirable. Such a survey would provide us with the background information and guidelines necessary to the design of future studies on isolated enzymes and enzyme systems.

METHODS

The Arctic ground squirrels (*Citellus undulatus*) used in this study were caught in the Little Susitna Valley of south central Alaska. While in captivity they were housed in individual wire cages and fed an ad libitum diet of whole kernel corn plus occasional lettuce and carrots. Water was available at all times. To induce hibernation, the animals were placed in a cold room (10 ± 1°C). At least 45 days were allowed to elapse before any hibernating or nonhibernating individuals were removed for study. As would be expected, the squirrels lost considerable weight during the course of the fall, winter, and spring studies. Thus, the weight range of individuals used in the fall was 628 to 825 gm whereas in the late winter or early spring the weight range was 305 to 430 gm. Five categories of individuals were selected: (1) before hibernation in the fall of the year; (2) one to three days hibernation; (3) 6 to 9 days hibernation;

[1] The views expressed are those of the authors and do not necessarily represent official Air Force policy. The experiments were conducted according to the "Rules Regarding Animal Care" as established by the American Medical Association (Air Force Regulation 160–94).

(4) one to two days after arousal from hibernation and (5) after completion of the hibernation season in the spring of the year. These animals were not segregated according to sex. However, no gross differences attributable to sex were observed in any of the measurements reported here.

The rats used for comparative purposes in these experiments were large (375 to 530 gm) adult males of the Sprague-Dawley strain. They were maintained in the animal colony at $26 \pm 1°C$ on an ad libitum diet of "Friskies" dog food and water.

In these studies, endogenous oxygen consumption was measured using tissue homogenates rather than tissue slices, since it was felt that over a long period of time more consistent and comparable homogenate than slice preparations could be obtained. For comparative purposes, measurements on large albino rats were also made. Four different tissues were studied: the cerebrum lateral to the lateral ventricles, a portion of the large median lobe of the liver, the apex of the heart ventricles and a portion of the large muscle mass from the thigh of the hind leg. The following procedure was used to prepare the tissues for study: First, the animals were killed by decapitation and the desired tissues quickly excised and chilled in chopped ice. Aliquots of tissue were then taken and weighed on a microtorsion balance and homogenates prepared in ice-cold 0.25 M sucrose to give a final tissue concentration of 3%. The homogenates were filtered and 1-ml aliquots transferred to Warburg reaction vessels for oxygen consumption measurements.

The assay medium in the endogenous respiration studies had the following final concentration of constituents: 0.002 M K-ATP (pH 7.4), 0.01 M K-phosphate buffer (pH 7.4), 0.0066 M $MgCl_2$; 5×10^{-4} M diphosphopyridine nucleotide; 1.5×10^{-5} M cytochrome c and 0.20 M sucrose. Where citrate oxidation was studied in liver tissue, substrate was added to a final concentration of 0.01 M. The final volume of the reactant mixture including tissue was 3 ml. The reaction flasks also contained 0.2 ml of 5N NaOH plus a folded piece of filter paper in the center well f the absorption of CO_2.

Following a 10 minute period of therm equilibration oxygen consumption mea urements were conducted for 60 minut at 38°C. The rate was calculated as micr liters consumed per milligram of nitroge per hour. Nitrogen was determined b nesslerizing an acid digested aliquot the filtered homogenate.

Due to wide differences in subclass va ances in the respiratory measurement Fisher's t test could not be legitimate used in many instances for a statistic analysis of the data. Statistical evaluatio was therefore made using the no parametric, Mann-Whitney U test (s Siegel, '56).

RESULTS

In the present experiments, the endo enous respiration of 4 tissues (brai heart, liver and skeletal muscle) we investigated at 5 critical periods of th yearly life cycle of the Arctic ground squi rel. The results of these experiments a presented in table 1. Also included in th table are the average rates for each of th experimental groups. A statistical sum mary of these data is included in table

Brain respiration. Cerebral tissue fro the fattened, nonhibernating Arctic grou squirrel had about 60% as great a respi atory rate as similar tissue from the ra In the squirrel the oxygen consumptio of this tissue was not appreciably altere during the first few days of hibernatio After 6 to 9 days in the latter state, ho ever, oxidative metabolism was depresse Thus, levels of approximately one-ha the prehibernation value were observe It was further observed that arousal fro hibernation produced little or no recove of the respiratory rate that characterize the prehibernating state. To a minor e tent such recovery of the initial rates di occur in the spring after the hibernatio season was completed.

Heart respiration. The effects of se son and hibernation on myocardial ox gen consumption were quite similar t those observed in brain tissue. Thus, sho intervals of hibernation were again wit out appreciable effect, and longer perio of hibernation were associated with marked reduction in respiratory rate. Wit

TABLE 1

Tissue respiration[1] during various stages of the yearly life cycle of the Arctic ground squirrel

N	Squirrel hibernation cycle					
	Before hibernation	1–3 Days hibernation	6–9 Days hibernation	Arousal	After hibernation	Rat
			Brain			
1	167	150	63	91	109	266
2	173	107	72	85	93	274
3	140	142	80	90	90	267
4	187	170		83	122	265
5	173	157			115	286
6	166	135				
Av.	168	144	72	87	106	272
			Heart			
1	336	391	118	170	197	582
2	292	348	123	167	209	503
3	281	305	133	187	189	601
4	359	276		179	184	635
5	278	267			215	550
		279				
Av.	309	328	125	176	199	574
			Muscle			
1	200	167	99	111	123	84
2	197	162	117	187	61	64
3	243	172	76	135	92	69
4	185	173	103	135	196	51
5	226	154				76
6	246					
Av.	216	166	99	142	118	69
			Liver			
1	108	61	49	112	110	50
2	105	64	66	108	100	59
3	106	89	79	111	119	55
4	112	58	53	104	99	47
5	110	51			142	46
		46				
Av.	108	62	67	109	114	51
			Liver citrate oxidation[2]			
1	352	195	187	282		209
2	349	229	158	275		187
3	307	190	199	289		232
4	295	234	198	290		176
5	321	200				199
Av.	325	210	186	284		199

[1] Respiratory rates expressed in μl O_2 consumed/mg tissue N/hour.
[2] Corrected for endogenous respiration.

espect to the latter, it was observed that ne myocardial respiratory rates after 6 o 9 days of hibernation were approxi-nately one-third of those observed in the onhibernating, or the 1–3 day hibernat-ng state. At least partial restoration of ne prehibernating oxygen consumption as again obtained during arousal periods nd in the spring after completion of the ibernation season. A point of special interest in these data on heart respiration, particularly as it might apply to the species variations in heart function at low temperature, was the markedly higher endogenous respiration exhibited by myocardial tissue from the rat as compared to squirrel. Thus, the rat exhibits almost twice as much respiratory activity as the prehibernating squirrel. Compared to the values obtained after long hibernation, after an

TABLE 2

Statistical summary[1] of tissue respiration data

Tissue	Hiber. cycle	1–3 days	6–9 days	Arousal	After	Rat
Brain (endogenous)	Before	n.s.	s	s	s	s
	1–3 days		s	s	s	
	6–9 days			n.s.	s	
	Arousal				n.s.	
Heart (endogenous)	Before	n.s.	s	s	s	s
	1–3 days		s	s	s	
	6–9 days			n.s.	s	
	Arousal				s	
Muscle (endogenous)	Before	s	s	s	s	s
	1–3 days		s	n.s.	n.s.	
	6–9 days			n.s.	n.s.	
	Arousal				n.s.	
Liver (endogenous)	Before	s	s	n.s.	n.s.	s
	1–3 days		n.s.	s	s	
	6–9 days			s	s	
	Arousal				n.s.	
Liver (citrate)	Before	s	s	s	—	s
	1–3 days		n.s.	s	—	
	6–9 days			s	—	

[1] Symbols used: n.s., not significant; s, significant, where $p \leq 0.05$ by the two-tailed Mann-Whitney U test (see Siegel, '56).

arousal from hibernation, or in the spring after the hibernation season is over, this species difference was even greater, i.e., three to 4 times higher rates in the rats than in the squirrels.

Muscle respiration. The seasonal and hibernation-associated changes in endogenous skeletal muscle respiration are also quite similar to those observed in the two preceding tissues. The only statistically significant difference to be noted in muscle was a 23% decrease in respiratory activity after the shortest interval of hibernation. As in the preceding tissues, however, the most marked reduction in oxygen consumption was obtained after longer intervals of hibernation. In the present instance, the rate of respiration after 6 to 9 days of hibernation is about one-half that observed before hibernation. Another basic difference between muscular tissue and brain and heart tissue was the comparative oxidative rates of squirrels and rats. Instead of the rat showing somewhat higher activities than the prehibernating squirrel just the opposite was observed; namely, rat muscle had about one-third the rate shown by squirrel muscle.

Liver respiration. In many ways, the responses of the liver tissue were quite different from the other three tissues studied here. For instance, it was the only tissue where minimal endogenous respiratory rates were observed during the first few days of hibernation; there being no apparent tendency for a progressive decline as hibernation progressed. Liver was also the only tissue where there was a complete recovery of the prehibernation rates during periods of arousal or at the termination of the hibernation season. Like muscle, but unlike brain and myocardial tissue, the endogenous liver respiration of squirrels was considerably higher than that observed in the rat.

In addition to measuring the endogenous respiration, the capacity of the liver to oxidize citrate was also tested. As would be expected, this substrate markedly stimulated the respiratory rate of the tissues. In spite of the stimulation, however, the various qualitative changes observed previously in the endogenous measurements were also quite apparent when citrate was added. The only statistical difference between the two experimental procedures was the failure of the tissue from the aroused animal to fully recover the respiratory rate shown by the tissue from the

hibernating animal when citrate was sent.

DISCUSSION

n the foregoing data, numerous sig- cant differences in endogenous tissue piration have been observed. In gen- l, these differences may be placed into ee categories: (1) those that are attrib- ble to season; (2) those that are attrib- ble to hibernation; and (3) those that attributable to species.

With regard to the first of these cate- ies, it was observed in the nonhibernat- squirrel that all tissues except liver ibited their maximal respiratory rates the fall just prior to the hibernation son. These high rates declined over the rse of the winter until minimal, or near imal, rates were obtained in the spring. s noteworthy that the intact hibernator s been reported (Kayser, '57) to exhibit ummer to winter decline in whole body tabolism that was similar to the tissue anges observed here. Kayser, Jacob and cot ('54), however, could not detect y comparable summer to winter change the respiration of kidney tissue.

The present experiments give no infor- tion on mechanisms responsible for se decreases in tissue respiratory rates r the course of the winter. Such infor- tion will only be provided by more de- led studies of the enzymes and enzyme stems. Accordingly, it will be necessary determine whether there is a decline the concentration of certain enzymes, decline in the concentration of readily idizable substrates, or a change in the ture of the substrates being oxidized different times of the year.

The second category of effects observed re were those attributable to hiberna- n. Here it was shown that the hibernat- g state, sooner or later, led to a decline the respiration of all the tissues studied. similar decline in endogenous respira- n has been reported for kidney tissue ayser, Jacob and Lucot, '54). It was o brought out by the present experi- nts that the magnitude of this effect s similar (about 40 to 60%) in all the sues studied. Finally, it was shown that ousal from hibernation was accompa- d by at least a partial recovery of en- genous tissue respiration.

Again, it might be asked whether the alterations in tissue respiration can be attributed to changes in enzyme concentrations, to changes in substrate concentrations or to changes in the nature of the substrates being oxidized. In these experiments at least a partial answer to this question can be supplied. Thus, it was shown in liver tissue that the addition of citrate as substrate, although markedly stimulating respiration, did not alter the basic qualitative effects of hibernation on tissue metabolism. Since the citrate was added in excess amounts the observed rates would presumably reflect the catalyic capacity of the tricarboxylic acid cycle for its oxidation. It would follow, then, that the decline in liver oxidative metabolism upon entrance into the hibernating state was most likely due to a decline in the activity or concentration of one or more of the enzymes and cofactors that make up the tricarboxylic acid cycle and the electron transport system. Similarly, the recovery of the respiratory rate during the arousal process would be due to a recovery of the particular enzymes or cofactors that were affected by hibernation.

The foregoing results give no indication of the nature of the substrates being oxidized by the hibernating or aroused animal. Respiratory Quotient measurements on the intact animal, however, seem to indicate that the hibernating state is associated with lipid oxidation while the aroused state is associated with a return of carbohydrate oxidation (Dogden and Blood, '54; Mokrasch, Grady and Blood, '60). It will be of interest in future experiments to determine whether the loss of tissue oxidative capacity is directly attributable to the shift between carbohydrate and fat oxidation or to some other, as yet unknown, factors.

The final category of effects observed here were those attributable to species differences in tissue respiration. These differences included higher rates of brain and heart respiration and lower rates of liver and muscle respiration in the rat as compared to the nonhibernating squirrel. In these measurements it was interesting to observe that the lower rates of respiration are found in the two tissues that retain readily observable functional activity

during the hibernating state. In contrast to this, the tissues, especially muscle, that are least active during hibernation have the higher respiratory rates. This would seem to be something of a paradox since we would expect more active tissues to exhibit higher rates of respiration. In heart muscle, at least, these species differences are probably located at the enzyme level. Thus, it has been shown (Covino and Hannon, '59) that the ground squirrel myocardium had much less capacity to oxidize a variety of substrates than the rat myocardium at both normal body temperatures (38°C) and at hypothermic body temperatures (15°C). In fact, glutamate was the only substrate where the oxidative capacity of the squirrel myocardium exceeded that of the rat. How these hibernating animals are capable of maintaining heart and brain function at low temperatures in the face of this reduced capacity for energy release presents a most intriguing question. What makes it even more intriguing is the fact that these animals, in spite of their lower rate of myocardial respiration, are able to maintain not only higher concentrations of adenine nucleotides than the rat, but are also able to prevent the breakdown of ATP during hypothermia (Covino and Hannon, '59). There is also evidence (Zimny and Gregory, '59) that they can maintain reasonably high levels of myocardial ATP at least during the early stages of hibernation.

SUMMARY

The effects of season (autumn versus spring), short and long term hibernation, arousal from hibernation on the endogenous respiration of cerebral, myocardial, hepatic and skeletal muscle tissue of Arctic ground squirrels were studied. The species differences in tissue respiration between laboratory rats and ground squirrels were also investigated.

1. In the ground squirrels, all tiss except liver exhibited higher rates of dogenous respiration in the autumn t in the spring. Liver respiration was u fected by season.

2. In all tissues, the hibernating s was associated with a reduction in enc enous respiration as measured at 38 This reduction was apparent after one three days in muscle and liver tissue a after 6 to 9 days in brain and heart tiss Arousal from hibernation produced a p tial to complete recovery of the prehiber tion respiratory rates. Experiments on capacity of the liver to oxidize citr suggested that these hibernation-indu changes in respiration were attributable changes in the catalyic capacity of tissue.

3. The brain and heart respiration w higher in the squirrel than the rat wh the converse was true for liver and mus tissue.

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me Aspects of the Electroretinographic Response of e American Red Squirrel, *Tamiosciurus dsonicus loquax*

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n the course of an investigation of the troretinograms of various members of family Sciuridae, some observations e made on the responses of the eye of American red squirrel, *Tamiosciurus lsonicus loquax,* which indicated that tain of its physiological reactions differ hewhat from those of other diurnal spe- s of this family. It was also found that re are slight but apparently significant ferences between the neural structure the retina of the red squirrel and that he ground squirrel.

t should be made clear at the outset t each of the diurnal species of the uridae so far examined have been found possess a pure-cone retina. This is true only for the ground squirrels (*Citellus* .) of which 6 have been studied to date, also for one chipmunk species (*Euta- as amoenus luteiventris*), for the prairie ; (*Cynomys ludovicianus*) and for three cies of tree squirrel, the American red irrel, *Tamiosciurus hudsonicus loquax,* European red squirrel, *Sciurus vulgaris* l the common gray squirrel, *Sciurus olinensis leucotis.* The retinae of the squirrels differ from those of the *ellus* species, the chipmunk and the irie dog in having two layers of cones tead of the more usual single layer, but erwise the general structure of all these e-cone retinae is very similar, such dif- nces as there are especially between ground squirrels and the American squirrel, being a matter of the relative nbers of cells to be found in the various lear layers. This probably means dif- nces in the amounts of differentiation Granit's ('47) sense) and summation between the *Citellus* retina and that of the red squirrel. The electrophysiological results to be reported here suggest that such differences really do exist.

In this paper we are reporting on the dark adaptation and flicker responses of the red squirrel as measured by means of the electroretinogram. The results are considered in relation to those obtained in similar experiments on two ground squirrel species, *Citellus lateralis* and *Citellus beecheyi* and one chipmunk species, *Eutamias amoenus luteiventris.*

METHODS

The preparation of the animals, anesthesia, placement of the electrodes, light stimulus and general experimental details have already been described (Tansley, Copenhaver and Gunkel, '60). The Grass electroencephalograph was used for recording the responses during the course of dark adaptation, but this instrument was too slow accurately to follow the responses to flicker. For these experiments, therefore, the responses were amplified and displayed on an ordinary cathode ray oscilloscope and photographed on moving film. The time constant of the amplifier was 900 msec.

For the dark adaptation curves the eye was adapted for 5 minutes to the full stimulus (7500 lux) with the room lights on. The room lights and the adapting light were switched off simultaneously and records were taken at measured intervals of about 5 seconds each for three minutes, using the full stimulus with a 1.2 neutral density filter and a flash duration of 250 msec. The actual timing at which

each stimulus was given was read off afterwards on the paper. Curves were constructed relating the changes in the *a*-wave, *b*-wave and off-effect to the period of dark adaptation. All changes were complete in three minutes.

For the flicker experiments a sectored disc was made giving equal duration light and dark intervals. The speed of the motor running this disc could be continuously varied to give stimulus frequencies varying between 17 and 170 flashes per second. A photocell placed between the disc and the eye was connected up so as to record on the second beam of the oscilloscope. The disc was then run at a speed slow enough to give an obviously intermittent response on the oscilloscope. The camera was then started and the speed of the disc increased until the response was quite steady. The actual speed at which fusion first took place was read off on the film after development. This procedure was repeated starting with the full stimulus intensity and continuing with increasingly dense neutral filters in the light beam until the stimulus was too small to evoke a response. The stimulus intensity was then increased by the same steps until its full intensity was again reached. The fusion frequency at each stimulus intensity was then read off from the film after development and the mean of the values for the two series "up" and "down" calculated. The crystalline lens was not removed in these experiments which were done on the dark-adapted animal.

When recording was complete the upper part of the animal was intravitally fixed through the ascending aorta after ligaturing the descending aorta in the chest and washing out with isotonic saline. Bouin's fluid was used for fixation. The eyes were then removed and left overnight in the fixative. The eyes were embedded whole in celloidin without removal of the lens and sections cut at 15 μ; they were stained with hematoxylin and eosin. All sections were cut at right angles to the elongated nerve head characteristic of the squirrel eye (Walls, '42) so that it was possible to choose comparable sections from the eyes of each species for comparison.

RESULTS

Dark adaptation curves. In figur the changes in the *a*-wave, *b*-wave off-effect of one of the ground squir *Citellus beecheyi*, during the first th minutes of dark adaptation are shown will be seen that all three waves incre in amplitude during dark adaptation though the change in the *a*-wave at stimulus intensity is very small. Figur gives the same curves for the red squir In this animal the off-effect decreases d ing dark adaptation; for the first few onds it is actually bigger than the *b*-wa but subsequently it gets smaller as *b*-wave increases and after about 10 onds its amplitude falls below that of *b*-wave. Some actual records from ot experiments on the same two species shown in figure 3. Here the records ir

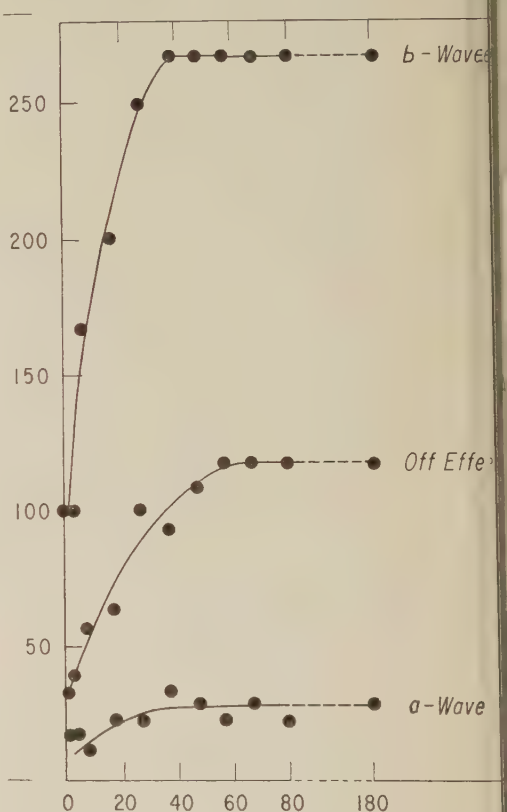

Fig. 1 Dark adaptation curves. The chan in amplitude of the *a*-wave, *b*-wave and off-eff of the ground squirrel, *Citellus beecheyi*, duri dark adaptation. Ordinates: potential develop in μv. Abscissae: time in the dark in secon

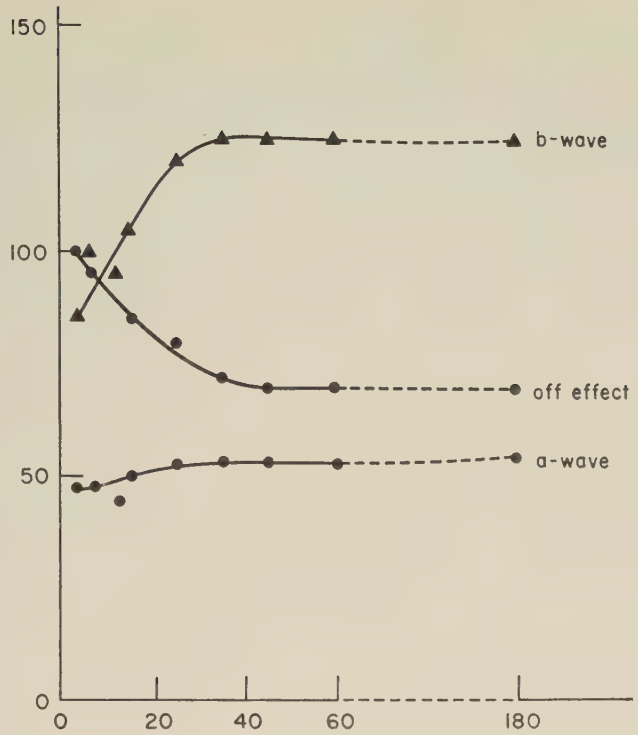

Fig. 2 Dark adaptation curves. The changes in amplitude of the *a*-wave, *b*-wave and off-effect of the American red squirrel, *Tamiosciurus hudsonicus loquax*, during dark adaptation. Ordinates: potential developed in μv. Abscissae: time in the dark in seconds.

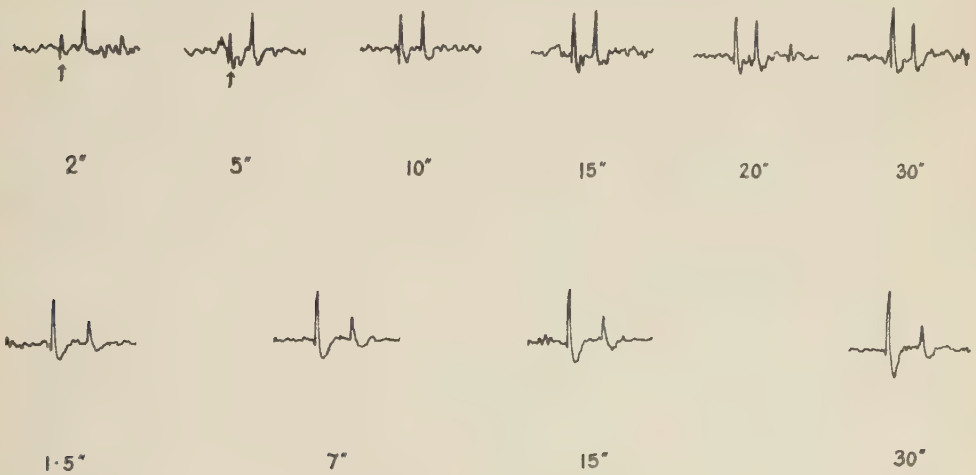

Fig. 3 Changes during dark adaptation. Responses during the first 30 seconds of dark adaptation. A, American red squirrel, *Tamiosciurus hudsonicus loquax*. The arrows at two seconds and 5 seconds mark the *b*-wave. B, Ground squirrel, *Citellus beecheyi;* 0.25 second stimulus in both cases. The paper speed was rather faster in B.

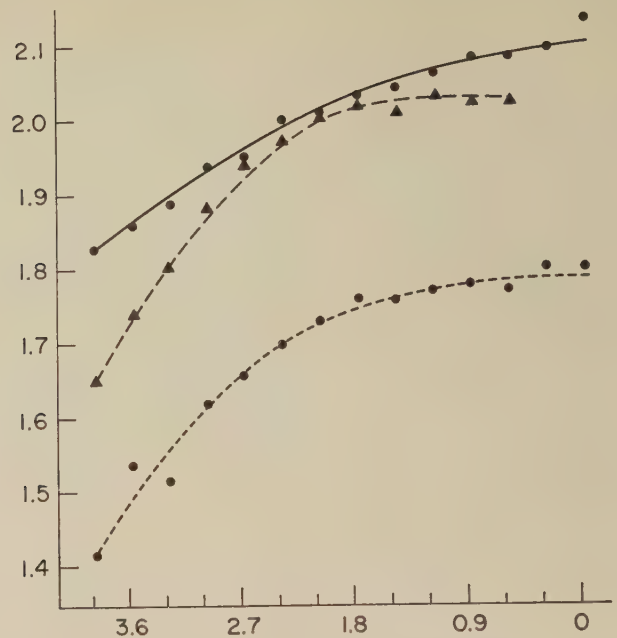

Fig. 4 Flicker fusion frequencies. The increase in fusion frequency with increased stimulus intensity. ●—● ground squirrel, *Citellus lateralis;* ▲---▲ chipmunk, *Eutamias amoenus luteiventris;* ●---● American red squirrel, *Tamiosciurus hudsonicus loquax.* Ordinates: log fusion frequency. Abscissae: neutral filter densities.

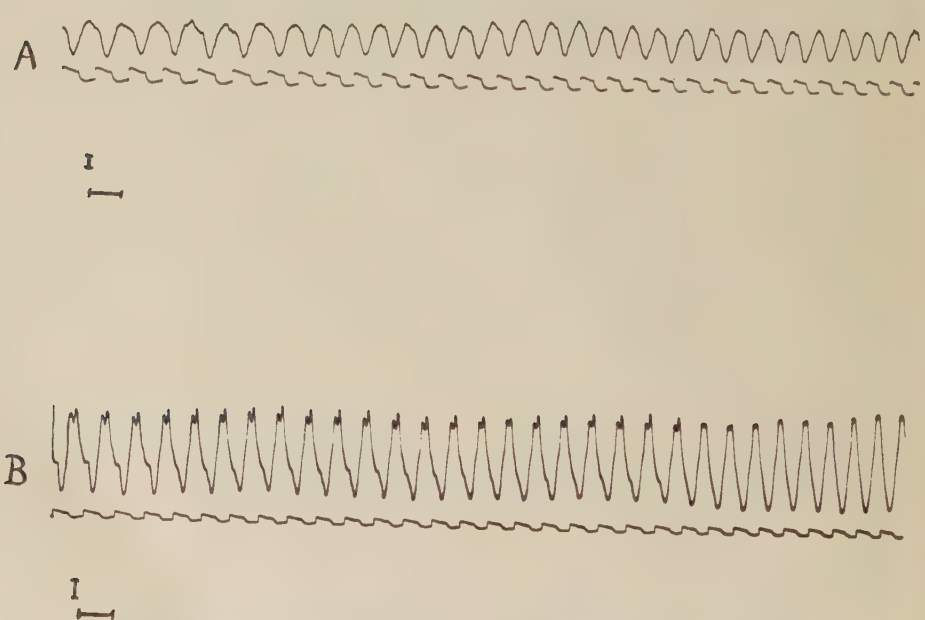

Fig. 5 Responses to flicker. A, American red squirrel, *Tamiosciurus hudsonicus Loquax.* B, Ground squirrel, *Citellus lateralis.* Calibration 100 μv. Time marking 50 msec. Stimulus intensity 7500 lux.

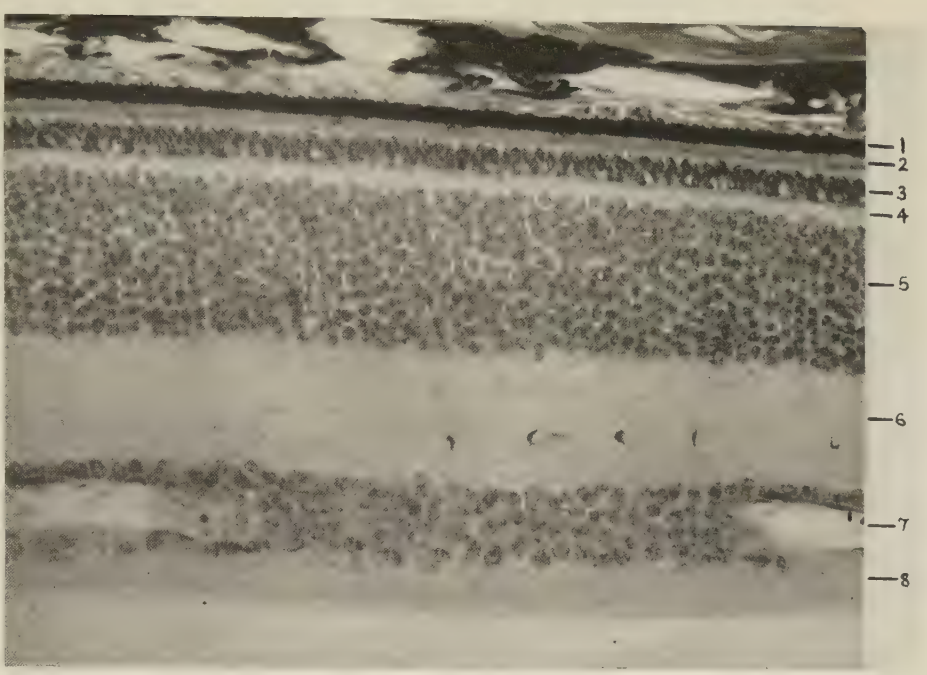

Fig. 6 Section through the central retina of the ground squirrel, *Citellus beecheyi*. Bouin. Hematoxylin and eosin. × 315. N.B. The following numbering applies to this and to figures 7 and 8. 1, pigment epithelium; 2, visual cells; 3, outer nuclear layer; 4, outer fiber layer; 5, inner nuclear layer; 6, inner fiber layer; 7, ganglion cell layer; 8, optic nerve fiber layer.

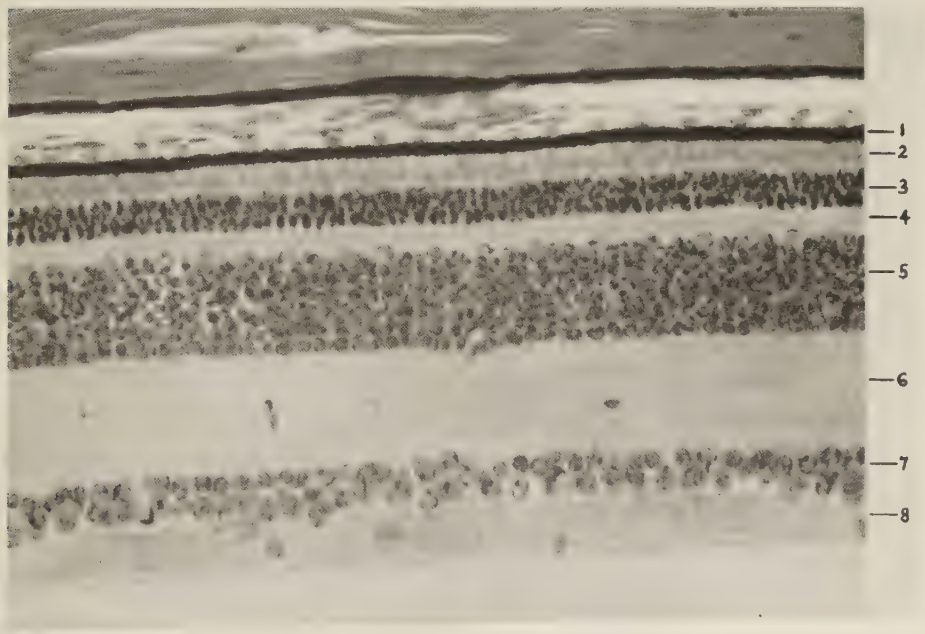

Fig. 7 Section through the central retina of the American red squirrel, *Tamiosciurus hudsonicus loquax*. Bouin. Hematoxylin and eosin. × 315.

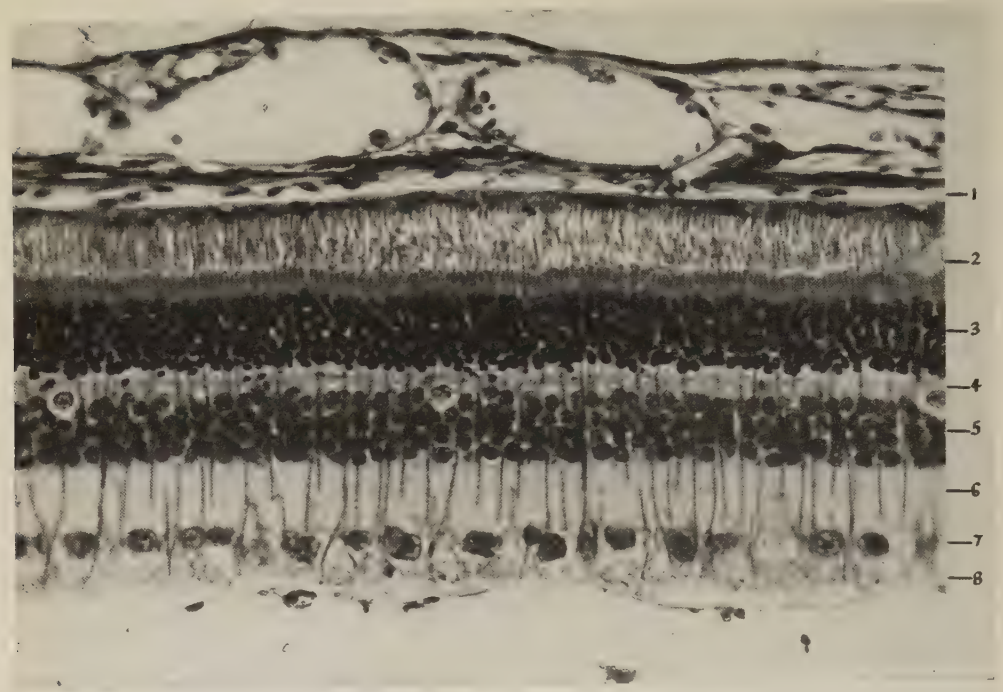

Fig. 8 Section through the central retina of the rabbit. Zenker. Mallory's phosphotungstic acid hematoxylin. × 294.

are from the red squirrel, those in B from the ground squirrel. In the latter the off-effect always remains smaller than the *b*-wave, but in the red squirrel the off-effect begins by being most obviously the bigger, after about 15 seconds the two deflexions are approximately equal and by 30 seconds the off-effect is definitely smaller than the *b*-wave. This was a constant finding for the red squirrel under the conditions of our dark adaptation experiments, but we never saw the effect in any other squirrel species. In all these the off-effect remained smaller than the *b*-wave and increased in amplitude during dark adaptation. In all these pure-cone retinae the changes due to dark adaptation were complete in 60 seconds or less. Such a rapid dark adaptation has already been reported for another pure-cone retina, that of the gray squirrel, *Sciurus carolinensis leucotis* (Tansley, '57).

Flicker. The curves in figure 4 show the increase in flicker fusion frequency with increased stimulus intensity for one specimen of the ground squirrel, *Citellus lateralis*, (top curve), a chipmunk (middle curve) and a red squirrel (bottom curve). All the ground squirrels and the chipmunk investigated gave fusion frequencies of between 95 and 120 per second with our maximum intensity stimulus, but we never obtained a figure above 65 per second for the red squirrel under the same conditions. This difference is undoubtedly due to the fact that the red squirrel's responses to light are slower than those of the ground squirrels. With our maximum stimulus the implicit (peak) time of the *a*-wave was 15 msec and that of the *b*-wave 35 msec for the red squirrel. The figures for *Citellus beecheyi* were 10 and 15 msec respectively with the same stimulus. Some records of flicker responses for the red squirrel (A) and *Citellus lateralis* (B) are shown in figure 5. These records also show how much more precisely the ground squirrel retina can follow the stimulus changes.

Histology. Figure 6 shows a section through the highly developed central retina of a *Citellus beecheyi* retina. This area forms a strip lying under the elongated nerve head and stretching right across the fundus. The unsually thick inner nuclear

and ganglion cell layers are very noticeable. The section from a red squirrel shown in figure 7 was most carefully chosen from a comparable part of the retina. It is shown at the same magnification. It is plain that neither the inner nuclear layer nor the ganglion cell layer are so well developed in this retina. A section through the central area of the scotopic (rod-dominated) retina of a rabbit is shown in figure 8 for comparison.

DISCUSSION

Our curves relating flicker fusion frequency to stimulus intensity in figure 4 are very similar in shape to those published by Bornschein and Szegvari ('58) for another ground squirrel species, *Citellus citellus*. These authors used an electronic flash of very high intensity and a very short duration, which was independent of the flicker rate, for their stimulus and, in consequence, their intensity range is quite different from ours. However, their maximum fusion frequencies are very nearly identical with those which we recorded for the chipmunk and the *Citellus* species. Our red squirrel curve, although similar in shape, is at a generally lower frequency level. All these curves obtained from pure-cone retinae are quite different from those reported by Dodt and Wirth ('53) and by Bornschein and Szegvari for the rod-dominated retina of the guinea pig. In this animal the curve is in two parts showing a scotopic maximum fusion frequency of about 20 per second and another at higher intensities when the fusion frequency can go up to about 50 per second. There is no sign of any such break either in our curves or in those shown by Bornschein and Szégvari.

As a result of his work on different types of retina, both rod-dominated and cone-dominated, Dodt has come to the conclusion that one can use the flicker fusion frequency as a means of differentiating between rod and cone responses. He believes that a fusion frequency above about 30 per second must be mediated by cones, and in a paper published in collaboration with Walther (Dodt and Walther, '58) uses the idea in the interpretation of some results obtained with the rabbit. The hypothesis may be correct although it is hard to understand how the comparatively small number of cones present in the rabbit retina can completely dominate the electroretinographic response in the way postulated by Dodt. Even if one assumes that the cones can inhibit the rod response as suggested by Elenius and Heck ('57) to explain some of their results in man, there would still appear to be too few cones in the rabbit to give a measurable electroretinogram on their own. If one accepts Dodt's hypothesis there is quite a lot of variation among the cone responses in different species with regard to the fusion frequencies they can achieve. Dodt and Wirth report a fusion frequency of 140 per second for the far from pure-cone retina of the pigeon using a one-to-one light-dark ratio at 8000 lux. These conditions are very like ours since we also used a one-to-one light-dark ratio at 7500 lux, but we never recorded so high a figure. And with these same conditions the red squirrel figure never went above 65 per second. So although the value for this animal falls within Dodt's range for cones it is very markedly lower than that recorded for other cone-dominated retinae under comparable conditions.

It is obvious that the relatively low fusion frequency of the red squirrel retina is a consequence of its slower responses to stimulation so that it is unable accurately to follow quick alternations of light and dark. But why should this particular pure-cone retina be slower in its reactions than the other pure-cone retinae of the squirrel family? There is no reason to believe that this retina contains rods in sufficient number to affect the electroretinogram. Leaving aside for the moment a fusion frequency which Dodt would consider to be well outside the rod range, the criteria already discussed (Tansley, '57) in connection with the visual cells of the grey squirrel also apply to the red. This retina shows no Purkinje shift and has a rapid and not very extensive dark adaptation. In addition its spectral sensitivity curve is the same as that found in the grey squirrel and in the ground squirrels and chipmunk (Tansley, Copenhaver and Gunkel, '60).

It seems more likely that the answer lies in the neural organization of the retina.

If we compare a section of the red squirrel retina (fig. 7) with one from a ground squirrel (fig. 6) it is obvious that the latter shows the more complex structure. Not only are there enough ganglion cells to give a one-to-one relation between visual cells and optic nerve fibers but there are many more cells in the inner nuclear layer. Such an increase in complexity is typical of a cone-dominated retina in comparison with a rod-dominated one as can be seen from the section of a rabbit retina shown in figure 8. This section is also from the central retina where ganglion and bipolar cells are most numerous. In this rod retina it is clear that there can be no question of a one-to-one relationship between rods and optic nerve fibers and that, so far from there being more inner nuclear cells than there are visual cells as in the cone retina, there are in fact many fewer.

Granit ('55) has shown that inhibition is much better developed in the cone-dominated than in the rod-dominated retina and he argues that this is the reason for the much faster responses of the former. Since one of the most striking differences between the two types of retina is in the number of inner nuclear cells it seems probable that the increase in these seen in cone retinae is associated with an increase in the amount of inhibition that can be developed. We do not know whether this increase in the number of cells in the inner nuclear layer is mainly due to an increased number of bipolar cells and, if so, of which types of bipolar. There is certainly some increase in horizontal cells and possibly also of amacrine cells. If we associate fast reactions with increased inhibition and this with an increased complexity of the inner nuclear layer then it would be natural to expect that the visual reactions of the red squirrel would be slower than those of the ground squirrel and that its flicker fusion frequency would be lower under comparable experimental conditions.

Another striking difference between the rod retina (fig. 8) and the cone retina (fig. 6) lies in the proportions of visual cells to ganglion cells. In the rod retina there are enormously more of the former and this must mean that a very large number of visual cells must be finally connected to each ganglion cell and so to each optic nerve fiber of which the ganglion cell is the nucleus. Such an arrangement provides for a high degree of summation of visual cell responses onto each optic nerve fiber and this seems to be the basis of the great sensitivity of the rod retina as compared to the cone retina in which there can be nothing like so much summation. A comparison of the red squirrel retina (fig. 7) with that of the ground squirrel (fig. 6) indicates that there is likely to be more summation in the former and one would therefore, expect the red squirrel to be more sensitive to low illuminations. On the other hand the ground squirrel retina should be capable of greater resolution owing to its possession of more ganglion cells and therefore of more optic nerve fibers. We have no information as to the relative visual acuities of the two species but it does appear that the red squirrel has a higher sensitivity. Whereas the ground squirrels and chipmunks are reported only to be active during the hours of full daylight, we observed that the red squirrel does not at all confine its activity to the hours between sunrise and sunset. Three individuals kept in an outdoor cage regularly played on a revolving wheel in the evenings through the twilight until nightfall.

We are unable to explain why the off effect in the red squirrel should be larger in the light-adapted state than during dark-adaptation when in the ground squirrel the reverse is the case. It is true that in the frog Granit and Riddell ('34) long ago showed that the off-effect is much increased during light adaptation. They also found that the purely negative response, produced by massage of the bulb and believed to be an expression of PIII activity, is increased by light adaptation and that it shows a quicker disappearance at "off" under these conditions. This was one of the findings considered by Granit when, in his well-known analysis of the electroretinogram, he suggested that the off-effect is largely due to the quicker disappearance of PIII than PII on cessation of illumination resulting in the transitory reappearance of a positive potential at "off." However, such an explanation cannot, as Granit and Therman ('37) first pointed

t and as has been shown much more re-
ntly by Tansley, Copenhaver and Gunkel
61), account entirely for the large off-
fect of the cone retina. There must, in
dition, be a reactivation of PII at "off."
may be that such a reactivation of PII
a more important component of the
ound squirrel off-effect while the inter-
tion of PIII and PII plays a greater part
the red squirrel. Some such hypothesis
ight expain the difference in response
dark adaptation of the off-effect of the
d squirrel as compared with that of the
her squirrel species.

SUMMARY

1. Some electroretinographic responses
the American red squirrel were com-
ared with those of two ground squirrel
nd one chipmunk species.

2. It was found that whereas the off-
ffect of the ground squirrel electroretino-
ram increased during dark adaptation
nat of the red squirrel decreased.

3. The flicker fusion frequency of the
ed squirrel as measured by the electro-
etinogram was consistently lower than
nat of the ground squirrels and chipmunk
nder the same experimental conditions.

4. The flicker results are discussed in
ne light of the retinal histology of the red
nd ground squirrels.

5. A tentative suggestion is put forward
o explain the different behavior of the
ff-effect in light and dark adaptation as
between the red squirrel and the ground
squirrels.

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eversible and Irreversible Changes in Normal uman Thrombocyte Membranes by Slow nd Rapid Swelling

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In previous communications (Gurevitch nd Nelken, '56; Gurevitch, Nelken and anon, '58) we described the morphologi- l changes occurring in normal human ood platelets when introduced into solu- ons of decreasing ionic strength. The rombocytes showed an increase in size; ey became swollen and the spicules be- me longer and broader. In the solutions very low ionic strength a peculiar rombocyte shape appeared; the throm- ocyte possessing a sword-like protrusion. ecently the osmotic fragility of normal uman blood platelets has been investi- ated (Nelken, Gilboa-Garber and Gure- itch, '60). The morphological changes of ne thrombocytes as well as the release of ne enzyme pyrophosphatase into the hypo- onic solutions were measured as a func- on of the decreased ionic strength. An nalogy could be drawn between the classi- al osmotic fragility test of erythrocytes nd the osmotic fragility of thrombocytes. n the osmotic fragility test of erythro- ytes the cells are directly introduced into ne hypotonic salt salution and the degree f hemolysis measured. When the hemol- sis is performed through the gradual de- rease in ionic strength (Danon, Nevo nd Marikovsky, '56) and the degree of emolysis measured (Klibanksy, '60), the esults obtained differ from those of the rst "drastic" method. The ionic strength t which the hemoglobin is released rough the red blood cell membrane in ne dialysis procedure is lower than in the rastic or classical hemolysis method. The ifference in the ionic strength at which ne hemolysis occurs when erythrocytes of ne same donor were examined by the dras- c and gradual methods is attributed to the visco-elastic nature of the erythrocyte membrane. The membrane is unable to resist the sudden pressure increase which occurs when the cell is introduced into a solution of low ionic strength. On the other hand, the red cell membrane withstands the slow increase of the internal pressure during the dialysis method down to a lower external salt concentration than in the drastic method.

In the present study an attempt has been made to examine normal human thrombocytes exposed to a gradual decrease in ionic strength as compared with thrombocytes exposed to "drastic" reductions in outer solution concentration.

MATERIALS AND METHODS

Preparation of thrombocyte suspension

The blood drawing, the anticoagulant solution and the method of separation of the thrombocytes have been described elsewhere (Nelken, Gilboa-Garber and Gurevitch, '60).

The decrease of ionic strength by the gradual method

Gradual decrease in the salt concentration surrounding the thrombocytes was achieved by dialysing a suspension of thrombocytes in physiological salt solution against the desired hypotonic solution in the following manner: Thrombocytes separated from 10 ml of blood were introduced into a dialysis bag[1] containing 10 ml of 0.15 N NaCl solution. The bag was suspended into a beaker containing 2000 ml

[1] Purchased from Fisher Scientific Company, New York, no. 8-667.

of 0.024 N NaCl solution. Another dialysis tube containing an equal volume of salt solution of the same concentration was suspended into the beaker at the same time. Every 5 to 10 minutes, 1 ml aliquot of the thrombocyte suspension was withdrawn and fixed in 1% (final concentration) osmium tetroxide in a NaCl solution of approximately the same ionic strength as the suspending medium of the aliquot, and buffered to pH 7.3 according to Palade '52. (Each batch of dialysis tubing was calibrated prior to performing thrombocyte experiments by establishing the decrease of ionic strength with time, under the experimental conditions stated.)

The "drastic" method

The "drastic" decrease in the salt concentration of the medium surrounding the thrombocytes was obtained by adding 0.05 ml aliquots of a concentrated stock suspension of thrombocytes in 0.15 N NaCl solution into 1 ml of NaCl solution taken from the second dialysis bag mentioned above (that containing no thrombocytes). As the solution in this dialysis bag may be presumed to have reached the same degree of hypotonicity as the one in the dialysis bag containing the thrombocytes undergoing gradual environmental changes, a direct comparison between the two methods under identical final conditions is possible. After 5 minutes in the hypotonic medium the thrombocytes were fixed by adding osmium tetroxide solution as described above.

After 20 minutes fixation, one drop of every suspension was examined between slide and cover glass under a phase-contrast microscope. One hundred cells were classified according to their different forms. The percentage of normal cells left in every sample as a function of ionic strength is shown in figure 1. The electron microscopical preparations were made by depositing a drop of suspension on a copper grid covered with a Formvar film. The drop was air dried, then washed with 10 successive drops of glass doubly-distilled water. After air drying the grids, they were shadow cast by platinum at a height to shadow ratio of 1 to 5. An RCA EMU 3B electron microscope was used.

Pyrophosphatase determination

The pyrophosphatase released from t thrombocytes into the surrounding Na solution was determined in 1-ml sampl according to a method previously describ (Nelken, Gilboa-Garber and Gurevitch, '6

Reversion to isotonicity

Gradual reversion to isotonicity w achieved by placing the dialysis bag co taining the remaining thrombocytes in lo ionic strength solution into 1000 ml 0. N NaCl solution.

Rapid reversion was accomplished b adding hypertonic NaCl solution to eac hypotonic salt solution containing th thrombocytes bringing it back to isoto icity.

Clot retraction experiments

The clot retraction was determined b adding 0.4 ml of the platelet suspensio to 0.4 ml of own plasma, 0.05 ml of 30% suspension of thrice washed erythrocyte and 0.1 ml of 4.5% $CaCl_2$. The clot retra tion was examined after three hours an 24 hours. (The erythrocytes were intro duced in order to facilitate the visualiza tion of the degree of clot retraction.)

RESULTS

I. The morphological examination

Phase-contrast microscope observations

The morphological examination with th aid of the phase-contrast microscop showed no fundamental morphological dif ference between the platelets in the dia ysis bag where hypotonicity was achieve gradually and the platelets introduced di rectly into the hypotonic solutions. When the NaCl concentration decreased to 0.1 N (after 10 minutes dialysis), an overall swelling of the platelets was observed. The same morphological changes, but more exaggerated, were seen in the platelets tha were directly introduced into the hypotonic solution of the same ionic strength.

When the salt concentration reached 0.10 N (after 20 minutes of dialysis) the swelling was more pronounced.

When the NaCl solution went down to 0.083 N (30 minutes dialysis) all the platelets from the dialysis bag were still with spicules, and may thus still be considered morphologically normal. Among those in-

ted directly into the same hypotonic ution, 8% of the platelets could be con- ered deformed, three of those 8% owed adjoining balloons, and 5% had veloped fine, long protrusions. The re- aining 92% of the platelets were still th spicules, and were counted as nor- al although they were very swollen.

At ionic strength 0.066 N (40 minutes alysis) the percentage of the swollen ough normal platelets was 92%, the re- aining 8% being thick, rounded plate- s of smaller diameter having no spicules. the platelets that were treated by the astic method to the same ionic strength, ly 58% were swollen normal cells. The st of the drastically treated platelets had tached ballons, or were thick, rounded atelets of smaller diameter without spic- es, some of them with swordlike protru- ons.

The greatest difference between the adual and the drastic method occurs at ionic strength of 0.058 N (50 minutes alysis). Of the platelets in the dialysis g 72% still had spicules, which were ghtly longer than those of the platelets an isotonic solution, but otherwise oked normal, about 20% were thick, unded platelets and the rest were plate- ts with attached balloons or with a vordlike protrusion. At the same salt ncentration, drastically achieved, only)% of the cells were morphologically nor- al, 34% were swollen up like balloons, % had developed swordlike protrusions, nd 53% were thick, rounded cells.

When the ionic strength reached 0.050 (60 minutes dialysis) only 25% were orphologically normal although some- hat irregular. Of the rest, 42% were atelets with attached balloons, 3% had vordlike protrusions, and 30% were ick, round platelets.

At the same solution in the drastic ethod only 5% of the platelets were mor- ological normal. Sixty-one per cent ere platelets with balloons and 37% ick, rounded platelets.

In the next solution (0.043 N) almost difference could be observed under the ase-contrast microscope between the atelets in the dialysis bag and those in- rted directly into the same hypotonic solution. In both cases about 87% of the platelets were balloons and 13% of them were rounded thick cells.

This comparative enumeration is summarized in figure 1 where the percentage of morphologically normal platelets in the gradual method is compared to that of the "drastic" method.

Electron microscope observations. These generally confirmed the results obtained by the phase-contrast microscope about the percentage of remaining normal cells at various salt concentrations. At an ionic strength of 0.066 N, however, a major difference, not observed in the phase-contrast microscope, becomes evident between the electronmicrographs of thrombocytes subjected to the gradual and drastic decrease in salt concentration respectively. A great number of torn and otherwise deteriorated membranes (figs. 4, 5) result in the drastic method, while very few of such damaged membranes appear in the gradual method in which the predominant picture is that of swollen but not torn thrombocytes (fig. 3). Another striking feature in the electronmicrographs is the evident increase in the size of the thrombocytes as the ionic strength goes down (fig. 6), and the recovery of the original size when the thrombocytes are brought back into physiological solutions (fig. 7). The damage to thrombocytes that had been brought down in ionic strength drastically (fig. 8) and then returned to physiological saline is much more apparent (fig. 9) than the damage caused to thrombocytes that were treated by gradual decrease in salt concentration (fig. 7). The method used for bringing back the thrombocytes to the isotonic medium, i.e., either dialysing the platelets in hypotonic medium against isotonic NaCl solution or adding sufficient amount of hypertonic solution did not make a notable difference. It should be noted that while the reversed cells from the gradual decrease did not look exactly like the original normal cells in isotonic solution, they recovered their spicules and normal size (fig. 7). The cells that were subjected to a drastic decrease in salt concentration rarely recovered their spicules and mostly were deformed (fig. 9).

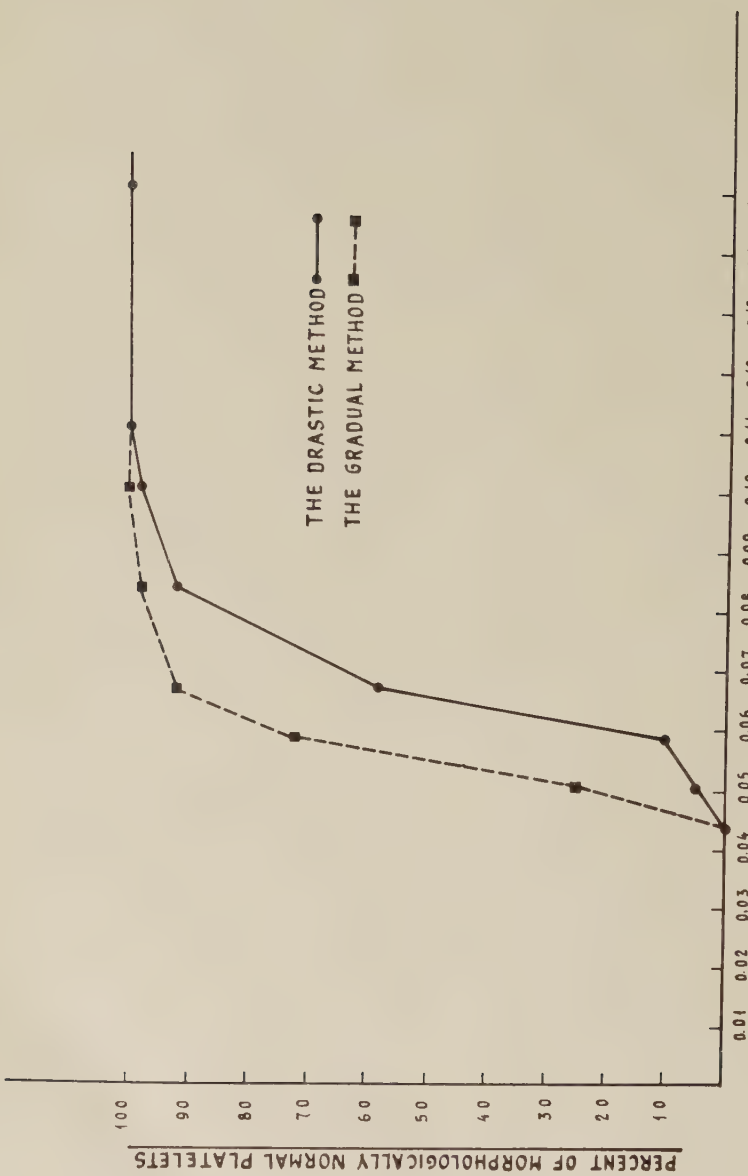

Fig. 1 The number of morphologically normal platelets left after decreasing the salt concentration by the "drastic" and gradual methods.

II. Determination of pyrophosphatase activity

Differences between the gradual and astic decrease in salt concentration treatents appeared also in pyrophosphatase lease from the thrombocytes. While ere was no release of pyrophosphatase om the platelets in the dialysis bag at ionic strength of 0.043 N NaCl, (a oint at which there was no longer any fference between the two methods in e phase-contrast countings (fig. 1), there as a considerable release of the enzyme om the platelets inserted directly into potonic solution of this concentration.

III. Clot retraction experiments

After bringing back the thrombocyte to otonic solution, their capacity to cause ot retraction was tested. It was found at no clot retraction activity was conrved by the thrombocytes treated by the astic method while the cells treated by e gradual method caused a partial clot traction. The method used for bringing ack the thrombocytes to the isotonic lution, by dialysis or by adding hyperic solution, did not make any difference the clot retraction capacity of the rombocytes.

DISCUSSION

The phase-contrast as well as the electron icroscope indicates that there is no basic orphological difference between platelets at have been subjected to sudden dease in the salt concentration of the rrounding medium, and those that have ached the low salt concentration very adually by the dialysis method. Apart om details, the modifications of the platets shape are similar in both methods. he percentage of thrombocytes of various apes, however, is different for the two ethods at corresponding salt concentraons. A certain fraction of the cells eated by the drastic method, moreover, resent a broken membrane. This is demnstrated on the one hand by the electron icrographs showing the damaged and oken membrane and on the other hand the leakage of pyrophosphatase. Anher significant difference between the vo methods appears when the ionic rength of the suspending medium is reversed to the physiological. Here, we see that the drastic method caused a total loss of the capacity of the platelets to bring about clot retraction, while the platelets that were treated by the gradual method partially conserve this capacity. The results obtained by the gradual method indicate that the membrane can endure large deformations without irreversible changes as long as these take place gradually. The reversibility of the platelet to a shape similar to its original as well as the conservation of its capacity of clot retraction is an indication of the rubber-like nature of its membrane. The molecular mechanisms in the membrane will be loaded by the stress and their ability to withstand the stress applied will depend on the rate of stressing. It seems that a certain time is necessary in order to bring about architectural changes on the molecular level in the membrane without breaking it. If this time is not available, as in the case of rapid swelling, the changes will be accompanied by disruption of the inter-molecular structure. Such behavior has already been attributed to the erythrocyte membrane (Klibansky, '60). In the thrombocyte, however, the return of the deformed membrane occurs over a wider range. The influence of the time during which the pressure is applied is also more apparent in the case of the thrombocyte membrane.

SUMMARY

Normal human thrombocytes were exposed to gradual and "drastic" decrease in the salt contration of the surrounding medium. Morphological changes of the thrombocytes were observed in the phase-contrast microscope and the numbers of morphologically normal cells remaining in the various hypotonic salt concentrations were counted and plotted as a function of the ionic strength. Electron micrographs are presented to illustrate the morphological changes caused by the two different methods. The release of pyrophosphatase enzyme is compared for the two methods as well as the clot retraction after return to physiological solutions. It was found that morphological changes resulting from the gradual method are reversible but that irreversible changes are produced by the "drastic" method. Pyrophosphatase is re-

leased only in the "drastic" method and partial clot retraction occurs only after return to the physiological condition in the gradual method.

ACKNOWLEDGMENT

The authors are indebted to Dr. O. Kedem and Dr. A. Silberberg for valuable discussions.

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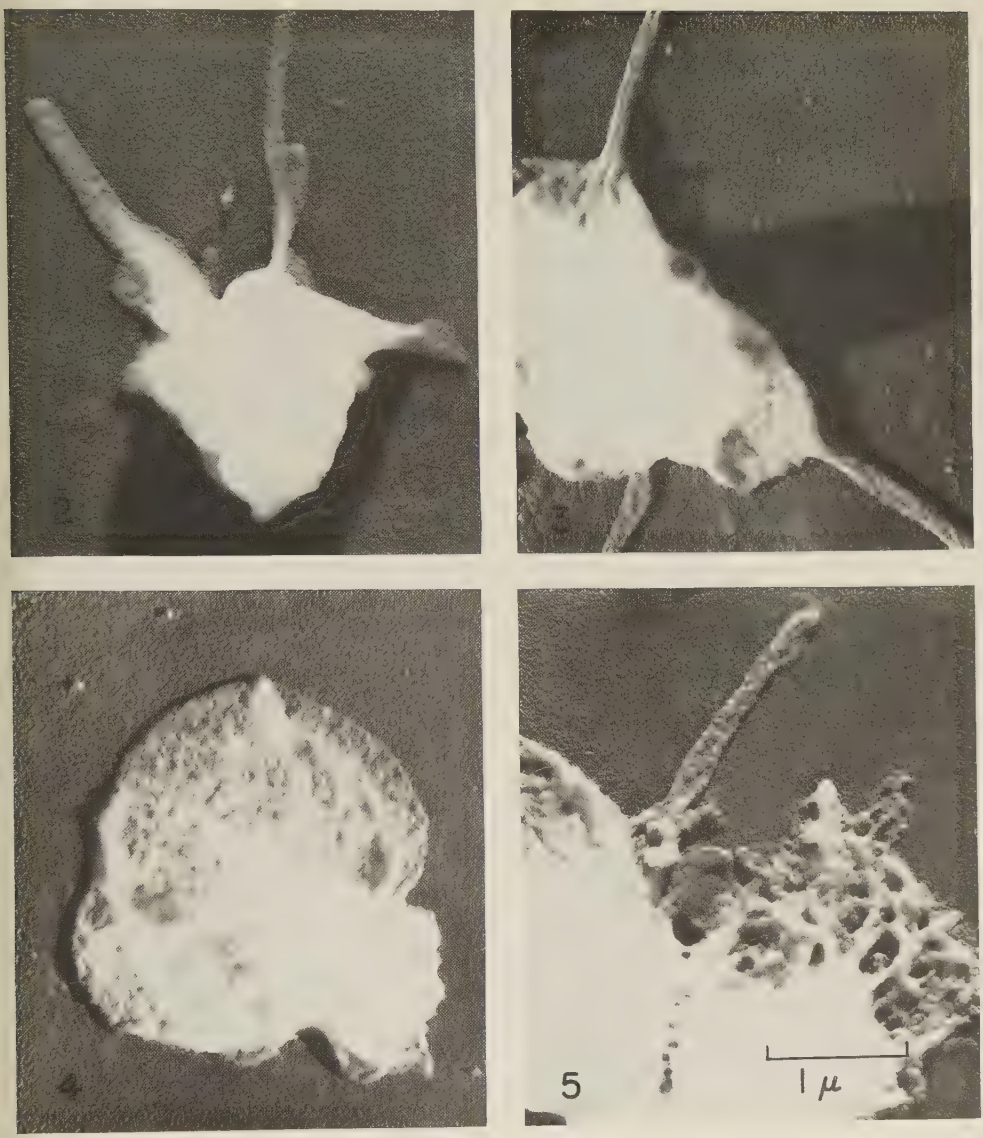
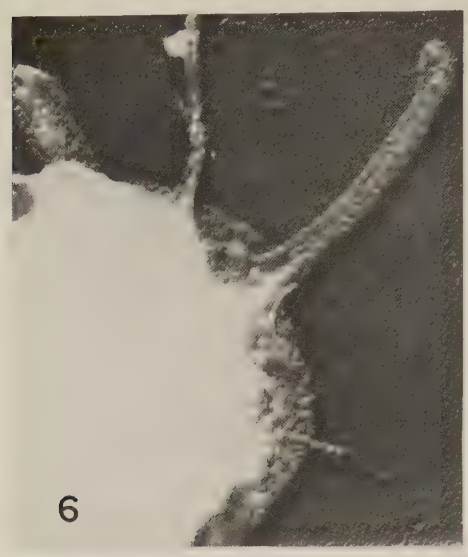


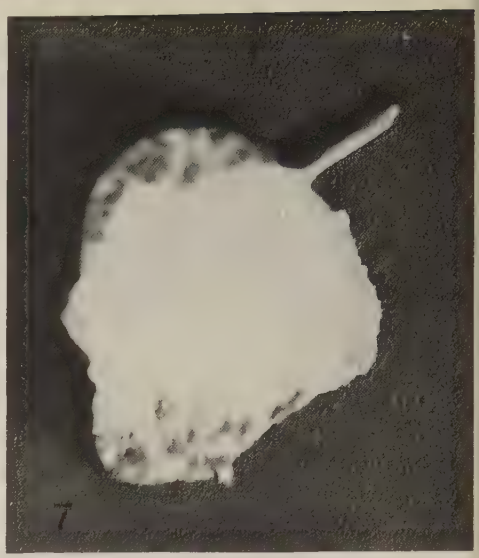
2 Normal human thrombocyte in 0.15 N NaCl.

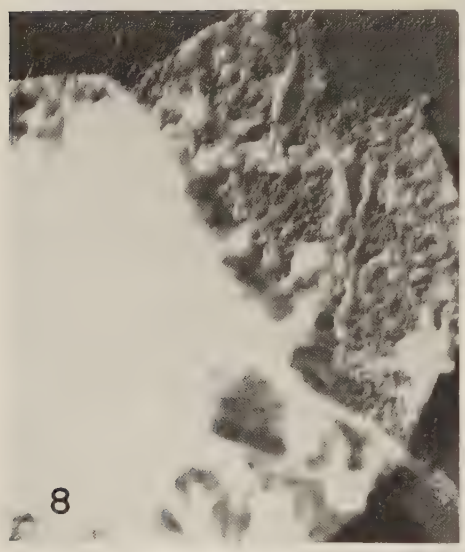
3 Human thrombocyte after gradual decrease in salt concentration of the surrounding medium down to 0.066 N NaCl (50 minutes dialysis).

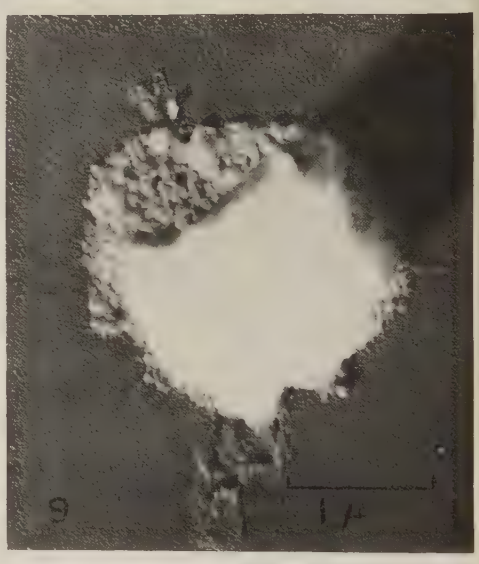
4 Human thrombocyte after sudden ("drastic") transfer into hypotonic solution of NaCl 0.066 N showing an adjoining balloon.

5 Same treatment as in figure 4 showing tears and holes in the thrombocyte membrane.









6 Human thrombocyte that was gradually brought by dialysis to the ionic strength of 0.043 N NaCl. Note the remarkable swelling.

7 Human thrombocyte that was gradually brought, by dialysis, to ionic strength of 0.043 N NaCl and then brought back to 0.15 N NaCl by adding hypertonic solution.

8 A thrombocyte brought down to the same ionic strength as in figure 6 but in a "drastic" method. Note tearing of the membrane.

9 A thrombocyte that was brought down by the drastic method and then back to physiological concentration. Note the deterioration of the membrane has persisted.

e Electrical Activity of the Amphibian Lymph Heart

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t is well known that the lymph hearts Amphibia lack the automatism char- eristic of vertebrate cardiac tissue. eir activity, like that of Arthropod rts, depends upon the integrity of their ve supply. In fact, each contraction the lymph heart is elicited by a volley nerve impulses that originate at a all group of spinal motoneurons (see ada, '56).

The electrical activity of the posterior mph hearts of the frog was studied by ücke and Umrath, and also by von lany, in 1930. Recording directly from lymph heart muscle these authors erved that each systole is accompanied a burst of electrical activity consisting 8 to 10 action currents with an overall ration of 0.2 to 0.5 seconds. The ampli- e of the recorded potentials was very due, presumably, to the shunting ef- t of the connective tissue in which the mph hearts are embedded.

Brücke and Umrath studied also the ntaneous electrical activity of the XI nal nerve,[2] the main source of motor nervation to the lymph heart. Bursts of ion currents, just above the limit of olution of the recording system, were wn to occur in this nerve. Although simultaneous electrical recording from th lymph heart and nerve was per- med, it could be concluded that the ion currents observed in the XI spinal rve were responsible for the initiation the periodic outbursts of electrical ac- ity recorded from the muscle tissue.

Since those papers were published, con- lerable advances have been made in both strumentation for electrical recording d our understanding of neuromuscular naptic mechanisms. It was interesting, erefore, to repeat some of the earlier rk with the techniques now available.

METHODS

Most experiments have been performed on the posterior, or coccygeal, lymph hearts of the toad *Bufo marinus*, which are comparatively large and flat, have rather thick walls and are situated just underneath the skin. The lymph hearts in the frog *Rana pipens*, were explored, but they seem to have a more irregular shape and are more deeply located. Tree-frogs *Eleutherodactylus portoricensis* (Schmidt), have also been used.

The toads and tree-frogs were anesthetized with urethane (2.5 gm/kg). This procedure results in more lasting preparations than those obtained by decerebration. Dissection was limited in most instances, to exposing the lymph heart by removing the skin and subcutaneous fasciae.

In the toad, the external surface of the lymph heart is surrounded by tough connective tissue which represents a nearly impenetrable barrier to the intracellular micropipettes. These electrodes could only be used on the inner surface of the organ. To do so the dorsal wall of the lymph heart was cut open. This procedure does not seem to interfere with the motor innervation as hearts prepared in this fashion went on beating vigorously for several hours.

Although the temperature of the laboratory where these experiments were performed was maintained between 19° and 25°C, the observed rates of the lymph hearts, of about 100 systoles per minute, were higher than those reported by other authors for the same temperature range

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[2] Called sometimes X spinal nerve, as an embryonic first spinal nerve is missing in the adult animal.

(from 60 to 70 per minute according to Braun-Menendez and Foglia, '40).

Extracellular recording. The electrical activity of the lymph heart was recorded with the aid of a pair of platinum wires (B. & S. gauge no. 24) covered by an insulating coat of varnish. Their exposed tips were applied against the dorsal surface of the heart. No difficulties due to movement artifacts were experienced. The gentle pressure exerted by the electrodes themselves was sufficient to immobilize the heart without producing noticeable damage to the tissues.

The potentials picked up by the platinum electrodes were differentially amplified by a Tektronix type 122 preamplifier and displayed on the screen of a Tektronix type 502 dual beam cathode ray oscilloscope. Photographic records were obtained with a Grass Kymograph camera. The preparation was grounded by means of a large electrode applied to the muscles of the thigh.

Intracellular recording. This was performed with the aid of conventional micropipettes, filled with 3 molar KCl solution and controlled with a Zeiss sliding micromanipulator. The metal electrode in contact with the KCl solution was connected to the Tektronix 502 CRO via a high input impedance head stage with low grid current and negative capacity neutralization (Lettvin et al., '58).

Recording of motor nerve impulses. Action currents of the XI spinal nerve have been recorded, after removal of the coccygeoiliacus muscle, by suspending the nerve from two platinum hooks in a pool of mineral oil. These electrodes were connected to a Tektronix type 122 preamplifier. As the number of motor axons in the XI spinal nerve supplying the lymph heart is small, not more than about 6 or 7, a high overall gain had to be employed in the recording system. The signal-to-noise ratio could be improved by dissecting from the main nerve trunk a small nerve bundle containing some of the desired motor axons.

Electrical stimulation. The electrical stimulation of the motor nerve fibers supplying the lymph heart could easily be achieved by applying pulses either to the spinal motoneurones or to the XI spinal nerve. Both these procedures have the advantage of resulting in the stimulat of many other motor axons and the cor quent mechanical and electrical artifa produced by the activity of surround muscles. Instead, by exploring the conn tive tissue surrounding the lymph he with a monopolar electrode, it was usua possible to find a site where weak electri stimuli gave rise to a contraction of heart without involving the adjacent sk tal muscles.

Recording of lymph heart movemen The movements of the intact lymph he have been recorded by a method develo in collaboration with Dr. J. Y. Lettvin. takes advantage of the fact that the m chanical distortion of the tip of a h resistance glass micropipette results marked changes in the electrical imp ance between the inside of the pipe and the external solution. So a mic pipette was placed upon the heart in su a position that its tip moved and be with each systole. The impedance of t micropipette tip was continuously mo tored by applying rectangular pulses current of constant strength and du tion of 2 msec. at a frequency of 2 cycles/sec. The voltage drop produced the current flow, which is proportional the impedance of the tip, was displa on the oscilloscope using a relatively sl sweep speed. An envelope was thus o tained representing the variations of t micropipette impedance. It is obvious th either an increase or a decrease of su impedance may accompany each syst depending upon the initial position of t micropipette tip and the pressure appli In figure 8 the micropipette was arran so that the contraction of the heart p duced an increase in tip impedance rep sented by an increase in the width of t envelope.

RESULTS

Extracellular recording from the lymph heart muscle

The records obtained with extracellul electrodes confirmed the results report by Brücke and Umrath ('30). Bursts electrical activity made up of a variab number of action currents, are seen ass ciated with each lymph heart contracti

both recording electrodes are placed n the lymph heart these action currents ear as diphasic variations (see fig. 1) se amplitude varies considerably not y in different preparations, depending n the magnitude of the external shunt, also from burst to burst in the same rt. The relative size of the positive and gative components of each action cur- t are also variable and depend mainly n the position of the electrodes on the sal wall of the lymph heart. The only nearly constant feature that one finds in these records is a somewhat slower monophasic wave at the end of each discharge, whose polarity is opposite to that of the main components of the diphasic currents.

The patterns formed by these action currents become simpler, more reproducible, and easier to interpret if only one of the recording electrodes is left in direct contact with the lymph heart muscle. The other lead may be placed on either a region of the heart previously damaged by

Fig. 1 Bursts of action currents recorded from a lymph heart by means of two platinum electrodes in contact with its dorsal surface. A heart systole is associated with each burst (see fig. 9). Notice the variability in the amplitude of the recorded currents and the duration of the intervals between consecutive systolic discharges.

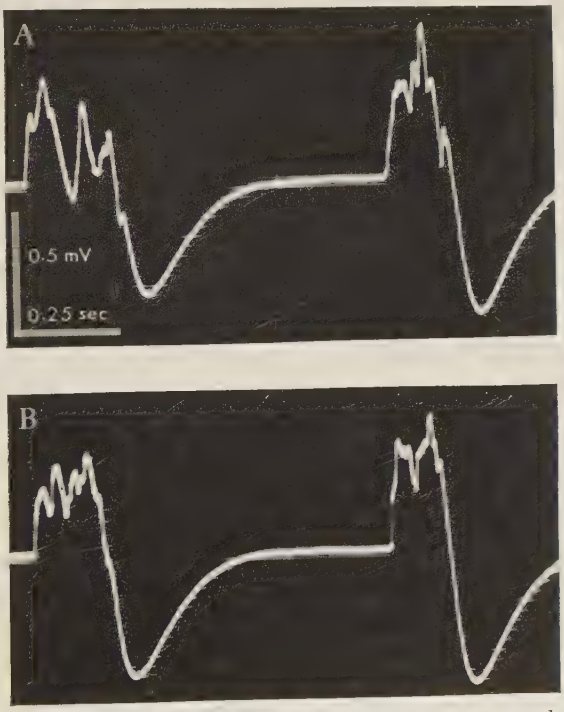


Fig. 2 Systolic discharges recorded from a lymph heart with external electrodes. Whereas in figure 1 *both* electrodes were in contact with the heart muscle, the above records were obtained by placing one electrode directly on the heart and the other on the surrounding connective tissue a few millimeters away. Calibrations in A apply also to B.

local heating, as Brücke and Umrath ('30) did, or in the surrounding connective tissue as we have done in most experiments.

In many preparations, the systolic discharges recorded in this fashion consist of two separate phases (see fig. 2). The first one is made up by a variable number of relatively fast waves (usually 5 to 10) that add up to produce an electrical variation of serrated or spiky contour. This is followed by a slower wave of smooth outline and opposite polarity whose amplitude is comparable to that of the first phase. It must be emphasized, however, that in some lymph hearts we have been unable to obtain such a clear-cut pattern of systolic discharge as those shown in figure 2. In those instances (see fig. 3) the recorded patterns seem to be intermediate between those shown in figures 1 and 2.

The polarity of the systolic discharges recorded with a couple of external electrodes in contact with the dorsal wall of the lymph heart depends upon the relative position of their tips. If the electrodes are radially oriented, the middlemost electrode becomes positive with reference to the peripheral one during the first wave each diphasic action current; indicat that this deflection is produced by curr flowing from the center to the periph of the heart.

Similar results have been obtained wh a single "active" electrode is placed up the center of the lymph heart. The re tively fast peaks that form the initial p of the systolic discharges are due to transient positivity of that electrode w reference to the "remote" electrode si ated outside of the lymph heart area.

If an intracellular microelectrode is serted into the lymph heart muscle ce (see later) one sees that the initial wav of the systolic discharge represent br depolarizations of their surface membra On the other hand (see last section Results), the externally recorded acti currents resemble the first derivative those potential changes.

This suggests that the externally corded positive deflections just discusse are due to current leaving the lymph c diac muscle cells across passive areas membrane; i.e., regions that do not parti pate in the permeability changes respo sible for cell depolarization.

Although no attempt has been made study in detail the distribution of sin and sources of current in the lymph hea sites have occasionally been found ne the edge of its dorsal wall, where acti currents of a polarity opposite to that records in figures 1 and 2 could be r corded. Exploring those sites with th platinum electrodes one could see th small displacements of the electrode t may result in a reversal of the sign of t recorded currents (see fig. 4). In betwee "positive" and "negative" areas the ele trode passes over points where the amp tude of the action currents appears great reduced.

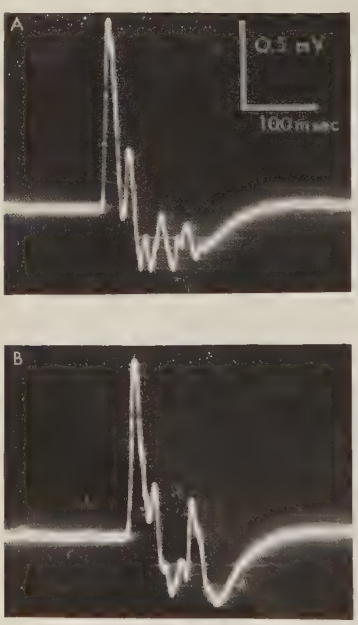


Fig. 3 Two systolic discharges recorded from a lymph heart with the same electrode arrangement as that used in figure 2 (see text). Calibrations in A apply also to B.

Electrical activity of the XI spinal nerve

As we have mentioned, Brücke an Umrath ('30) demonstrated the existen of spontaneous bursts of action curren in the XI spinal nerve of the frog whic occur at the same frequency as the lymp heart systoles. The lymph heart contra tions stop as soon as the nerve supply

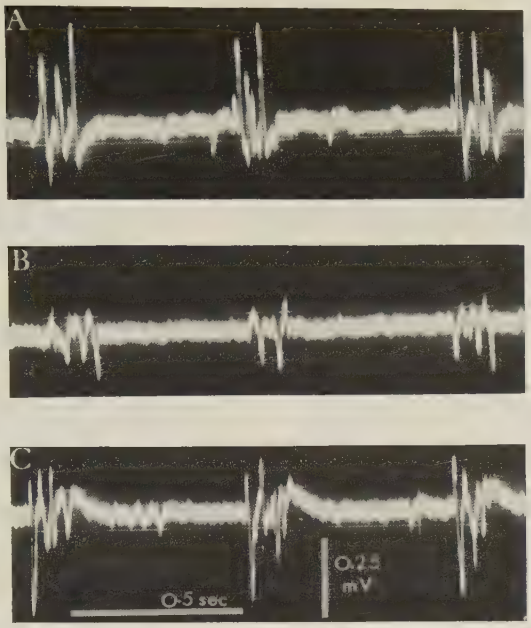


Fig. 4 Systolic discharges recorded from the dorsal surface of the lymph heart with a thin platinum electrode (0.1 mm in diameter). Its position was shifted from record A to B, and from B to C, in two steps of about 0.7 mm each. Notice changes in the patterns formed by the action currents (see text). Calibrations in C apply to all records.

errupted although discharges of action rrents go on in the central portion of e cut nerve. This indicates that the ntaneous nerve activity represents the lleys of motor impulses which elicit each stole. It was, therefore, interesting to ord simultaneously the electrical activ- of both nerve and heart to observe more sely their relationships.

For this purpose, the electrical dis- arges of both nerve and lymph heart re picked up by platinum electrodes and splayed on separate beams of the CRO. could then be seen how each burst of uscle action currents is associated with train of nerve spikes.

If the entire nerve trunk is suspended m the electrode, the amplitude of the tor spikes is barely higher than the base e noise. However, it is possible to in- ease the relative magnitude of the sig- ls by dissecting small bundles of fibers m the XI nerve placing them separately on the recording electrodes. In this shion preparations can be obtained con- ining a few lymph-cardiac motor axons ich give relatively large spikes. Records made from these preparations are illustrated in figure 5. The correspondence between nerve and muscle activity is very obvious in some systolic discharges. In record 5F, for instance, each muscle action current is associated with spike activity in the bundle of fibers placed upon the nerve electrodes. In some records such as 5D, a certain correlation seems to exist between the number of nerve spikes and the amplitude of the corresponding muscle action currents. In record 5A, however, we can see a muscle action current (the first one) which is not accompanied by motor nerve impulses. Conversely, nerve spikes are seen which are not followed by muscle action currents. This shows that the nerve impulses observed are only a fraction of those that arrive at the lymph heart, while the recorded muscle action currents represent the activity of only a part of the organ. It is clear, however, that each fast deflection recorded from the muscle is preceded, and presumably elicited, by a variable number of motor impulses reaching the lymph heart along several axons. These impulses are not

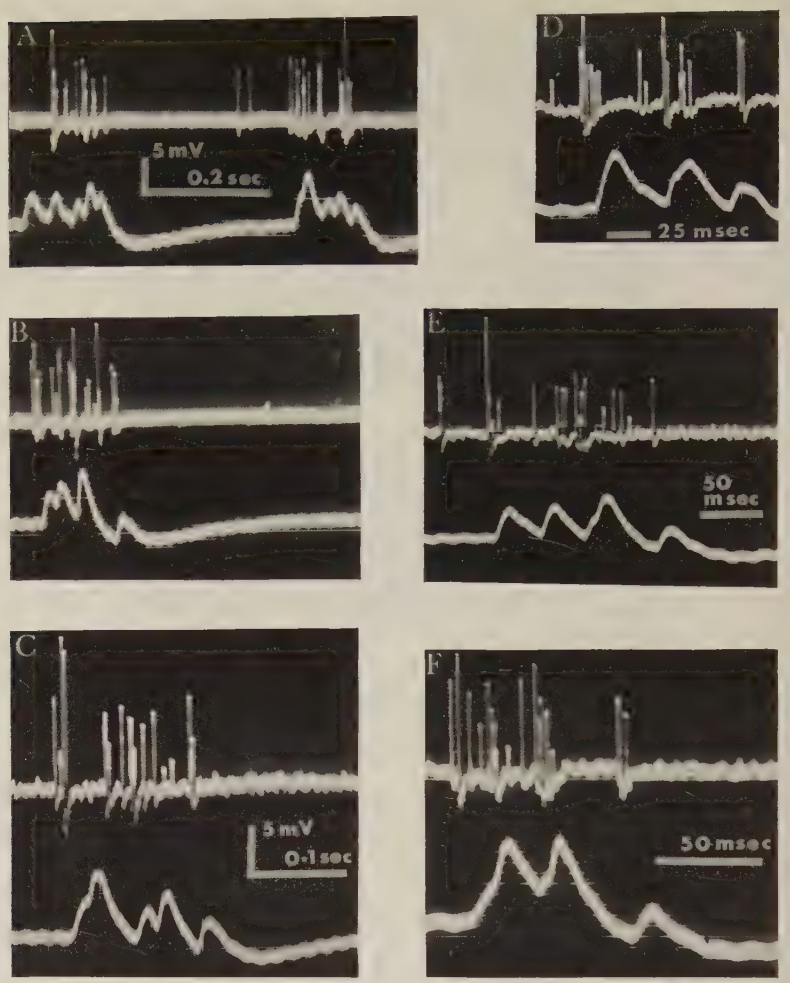


Fig. 5 Simultaneous recording of lymph heart muscle action currents (lower trace) and spontaneous electrical activity in a bundle of nerve fibers dissected from the XI spinal nerve (upper trace). The nerve spikes represent the motor impulses responsible for the synaptic activation of the lymph heart muscle. Voltage calibration in record A applies also to B, D and E, and that in record C applies also to F (lower traces only) time calibration in A applies also to record B.

synchronous, although all of those associated with a single muscle action current seem to arrive at the lymph heart within a period not longer than 12 to 15 msec., see for instance record D in figure 5.

Electrical stimulation of lymph-cardiac motor axons

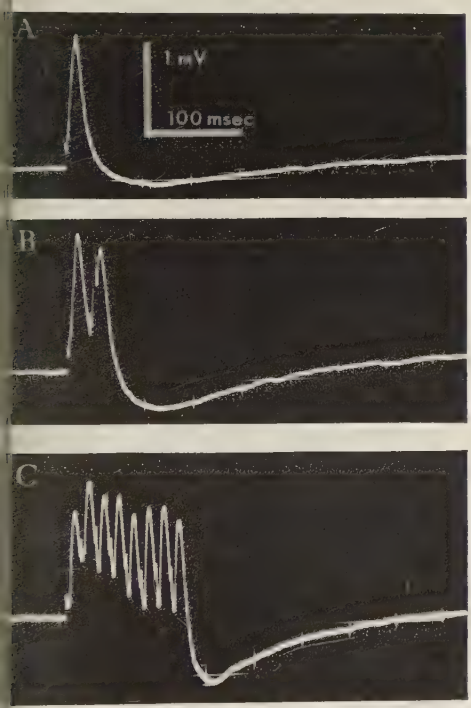
Another way of obtaining information on the relationship between nerve and muscle activity at the lymph heart is to stimulate directly the lymph-cardiac motor fibers. The easiest method to do this is t explore the surrounding connective tissu with a monopolar stimulating electrod Weak negative electric pulses are applie between the tip of this electrode and larger one making contact with the mu cles of the thigh.

Although the anatomical course fo lowed by the motor axons is variable, site could be located in most preparation where the applied pulses gave rise to co tractions of the lymph heart not accom

nied by twitching of adjacent skeletal scles.

Figure 6A illustrates the effect of a sin- supramaximal pulse applied to the nph-cardiac motor axons. The stimulus, rked by the artifact, is followed after a ef delay by a diphasic action current nsisting of a relatively fast initial phase ntinued by a slower deflection of op- site polarity. These two phases will be erred to as *fast* and *slow*.

The overall duration of the fast phase, , from its beginning to the point where e baseline is crossed, is of about 40–45 ec.; the time of rise to peak accounting approximately one third of this period. e leading edge of the fast wave often nibits some irregularities which might due to differences in the excitation + nduction time of the various motor ons.



ig. 6 Action currents of the lymph heart scle elicited by direct electrical stimulation of nerve supply (see text). In A a single stimulus s applied whereas B and C show the effects of nd 8 stimuli respectively. In record B the in- val between the two stimuli is of 25 msec. The ervals between the stimuli in record C are 20 ec. Calibrations in record A apply also to B l C.

The overall duration of the second or slow phase is of over 0.25 sec. (at a temperature of 23°C); that is, about 6 times longer than the fast or initial phase. The time of "rise" of this phase, to a rather ill-defined peak is of about 75 msec. at the same temperature.

If two pulses are applied in rapid succession (see fig. 6B), the double peak formed by the partial summation of the fast waves elicited by each stimulus is followed by a single slow wave of the opposite sign. No indication of the individual "slow" phases can be distinguished as steps or notches; the single slow wave shows, indeed, a very smooth and uninterrupted contour.

Figure 6C illustrates the effect of applying 8 consecutive stimuli to the lymph heart motor fibers. The resulting pattern of action currents is very similar to some of the spontaneous systolic discharges. Another example of fast repetitive stimulation of lymph-cardiac motor fibers can be found in figure 8B.

Figure 7 has been drawn by superimposing tracings of separate records showing the lymph heart action currents elicited by 1, 2, 3, 4 and 5 stimuli applied to the motor axons. The interval between consecutive stimuli was of 20 msec. The insert shows the maximum amplitude of the second or slow phase in each record plotted against the number of applied stimuli. It can be seen that the amplitude of the slow wave following 1, 2, 3, or 4 fast deflections is equal to the sum of an equivalent number of slow waves following single stimulus.

The polarity of the action currents elicited by direct electrical stimulation of the lymph heart nerve supply depends also upon the position of the "active" recording electrode within the lymph heart area. This polarity is identical with that of the spontaneous systolic discharges recorded with the electrode in the same position. This can be shown by stimulating the motor axons in preparations with an intact central nervous system and lymph-cardiac nerve supply. By doing so, it is possible to record side by side, the muscle action currents generated by motor impulses originating at lymph-cardiac spinal motoneurones and those elicited by the

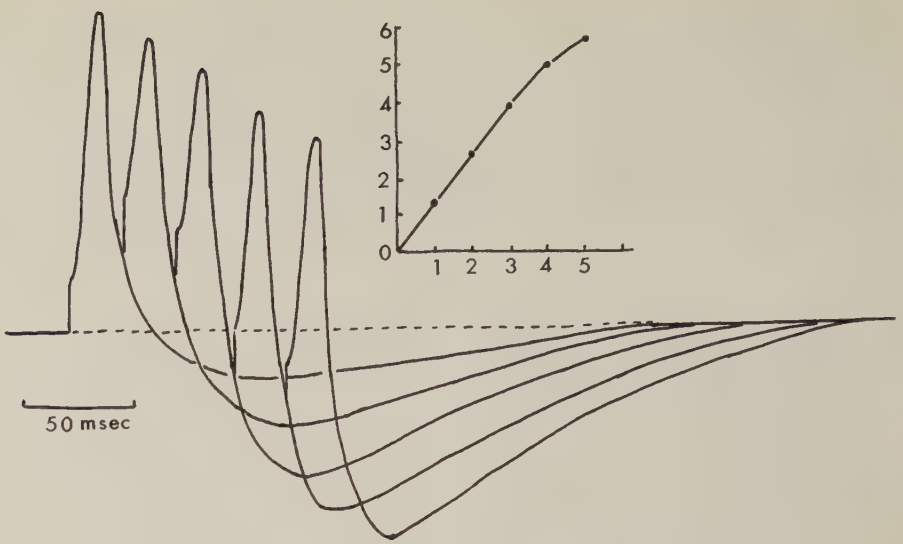


Fig. 7 This figure has been drawn by superimposing tracings of separate records showing the lymph heart action currents elicited by 1, 2, 3, 4 and 5 stimuli applied to the motor axons at a frequency of 50 per second. The insert shows the maximum amplitude of the slow phase in each tracing plotted (ordinate) against the number of applied stimuli (abscissa).

electrical stimulation of their axons in the periphery. This is illustrated in figure 8.

Relationship between the electrical and mechanical activity of the lymph heart

It is easy to see that each systole of the lymph heart is associated with a burst of electrical activity. However, to establish more precisely the relationship between these two events, experiments have been performed in which the movements and the electrical activity of the lymph heart were displayed separately on each beam of the CRO.

By recording the changes of the impedance of a micropipette applied against the dorsal surface of the exposed lymph heart an overall picture of its movements can be obtained. The records resulting from these experiments, some of which are illustrated in figure 9, show that the amplitude of the displacements of the lymph heart tissue is not constant. Very often, furthermore, systoles are observed in which the rising phase of the contraction is interrupted by a step or even by a brief period of relaxation. These patterns are invariably associated with a clustering of the corresponding action currents into two distinct groups separated by a brief silen period. However, when the action current form compact patterns such as those see in figure 9, the ensuing contractions hav smooth and uninterrupted contours an single well-defined peaks. The foot, or be ginning, of the contraction appears approximately 23 msec. (average of 4 contractions) after the initiation of th systolic discharges. The peak mechanic displacement occurs generally at the en of the systolic discharge, just before th recorded potential returns to the baselin

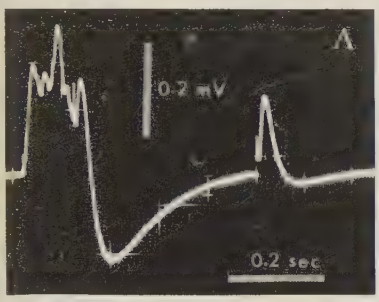
After the peak of the contraction reached, relaxation takes place at a ra that soon reaches maximal value and di cays before reaching the presystolic leve In most records the relaxation proceed until the beginning of the following sy tolic contraction.

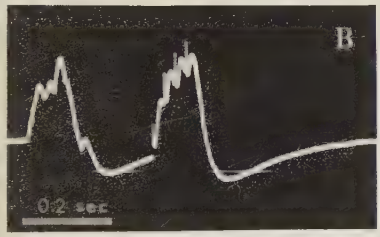
Action of some drugs upon the lymph heart

The influence of different compounc upon the lymph heart has been studied b several investigators; their findings, sum marized by Braun-Menendez and Fogli ('40) (see also Priestley, 1878, for refe ences to early work), show that this orga is particularly sensitive to drugs acting o

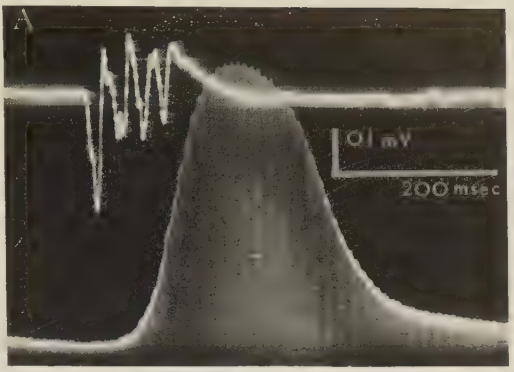
linergic junctions, such as nicotine, scarine, d-tubocurarine (dtC), acetyl-line (ACh) and other related sub-nces.

We have repeated some of those experi-nts and have confirmed the main re-s. Our observations have shown, how-r, that drugs are active on the lymph rt at concentrations significantly lower n those reported in previous papers. instance, dtC said to block the lymph rt contractions at a concentration of $^{-2}$ w/v (see Foglia and Braun-Menendez,) is still active if applied locally at con-trations of 10^{-5} to 10^{-4} w/v. ACh is active at similar concentrations. This crepancy can probably be explained by





ig. 8 Records showing systolic discharges erated by spontaneous neural activation of the ph heart together with action currents elicited electrical stimulation of lymph-cardiac motor ns in the periphery. In A, an action current duced by a single shock applied to the motor ns (easily recognizable by the stimulus arti-t) appears, by chance, at the end of a spon-eous systolic discharge made up by at least istinct peaks. A single action current elicited electrical stimulation is seen in the same ep. Notice the marked difference in the plitude and time course of the two action rents elicited by artificial stimulation (see cussion). In B, a spontaneous systolic dis-rge made up by at least 5 action currents urs just before a rather similar pattern elicited 4 consecutive electrical stimuli. Voltage cali-tion in record A applies also to B.



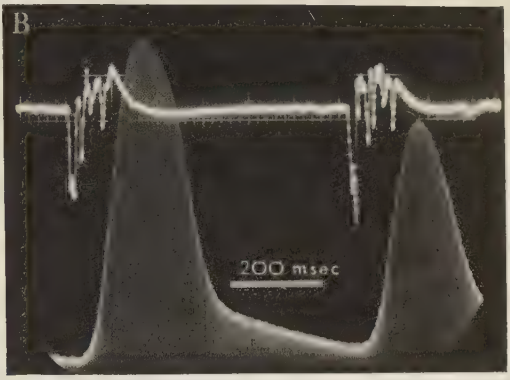


Fig. 9 Simultaneous recording of the electrical activity (upper trace) and movement (lower trace) of the lymph heart muscle during spontaneous systolic contractions. Voltage calibration in A applies also to B.

the fact that we have applied the drug solutions directly on lymph hearts after cutting open their dorsal wall. If the lymph heart is intact, the diffusion of the compounds into the muscle tissue is likely to be hindered by the very dense connective layers which cover its dorsal surface. This difference in technique might also explain that although atropine at concentrations as high as 10^{-2} w/v had been reported to be inactive (Foglia and Braun-Menendez, '39; Hotovy, '39) we have found that a block of the heart contractions is produced by atropine sulfate at a concentration of 10^{-3} w/v.

While working with atropine solutions we have confirmed one of the observations reported by Brücke and Umrath ('30); namely, that a lymph heart blocked by ACh at a concentration of 10^{-4} w/v

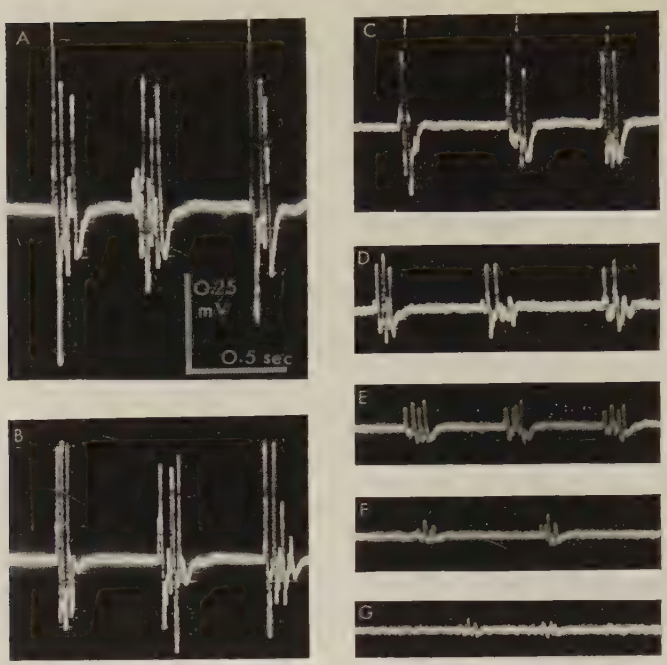


Fig. 10 Effect of d-tubocurarine upon the electrical activity of lymph heart muscle. A, normal systolic discharges used as control. After record A was taken 10 mg/kg of d-tubocurarine were injected intraperitoneally in the toad. Records B, C, D, E, F and G were taken 10, 30, 50, 70 and 80 minutes, respectively, after the injection of the drug. The amplitude of the recorded action currents shows a gradual decrease until they merge into the base line noise. No significant changes in the patterns formed by them can be distinguished (see text). Time and voltage calibration in A applies to all records.

starts beating again if atropine, at the same concentration, is added even if the initial concentration of ACh is maintained throughout the experiment.

The electrical activity of the lymph heart muscle under the influence of dtC and ACh was also examined. Particular attention was paid to the effect of d-tubocurarine on the lymph heart action currents. If the diphasic action current (fig. 6A), that may be regarded as the basic pattern of electrical activity of the lymph heart muscle, represented a combination of an excitatory post-synaptic potential plus a superimposed action potential, one would expect that the block produced by dtC would help to distinguish between those two types of phenomena. These expectations were not fulfilled. If one examines the electrical activity of the lymph heart while a dtC-block is established one sees how the amplitude of the muscle action currents decreases gradually until they merge into the baseline noise (fig. 1
We have not been able to find either d
continuities or marked changes in t
shape of the recorded action currents, su
as those observed during curarization
skeletal nerve - muscle preparations
which muscle action potentials give pla
to end-plate potentials.

Similar results have been observed aft
the intraperitoneal injection of lar
amounts of ACh (1 ml of a 2×10^{-2} w
solution). In other experiments, solutio
containing ACh at lower concentratio
(10^{-4} w/v) have been applied directly
the open lymph heart while its electric
activity was being recorded. Under the
conditions ACh caused not only a mark
decrease in the amplitude of the actio
currents; in some instances the polari
of the systolic discharges was reversed.

These results can be explained on t
basis of a differential action of ACh up
the lymph heart cells, due presumably

e existence of diffusion barriers. The ore accessible synapsis will be blocked st. This will alter the distribution of ources" and "sinks" of current in the sue.

It must be emphasized that although a $^{-4}$ w/v solution of ACh did not completely block the systolic discharges of tion currents the corresponding potential changes in the cell membrane were obviously too weak to elicit an appreciable mechanical response.

Intracellular recording

The lymph heart offers an unfortunate combination of factors that hinder the use of microelectrodes: small cell size, tough connective tissue, and extremely

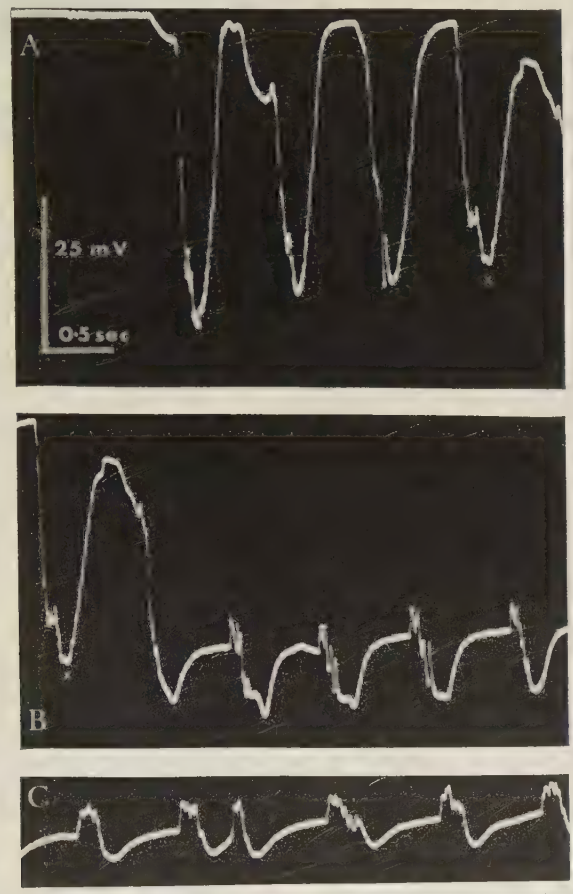


Fig. 11 This figure illustrates the potentials observed during a slow impalement of a lymph heart muscle cell with a micropipette and also the electrical activity of the cell membrane associated with each systole. The microelectrode is shifted steadily towards the muscle tissue from the beginning of record A until the end of B. Both records overlap and the deflection marked with an X in A and B is the same one.

The trace in the upper left corner of A shows the *zero* level; i.e., the potential recorded when the micropipette tip is in the external solution just outside the cell membrane. The large downward deflections that follow (indicating a negativity of the micropipette) represent transient records of the resting potential due to momentary penetrations of the microelectrode into the cytoplasm of the muscle cell. Eventually, in record B, the microtip stays in the cell and the changes in membrane potential associated with each systole can be observed. The last deflection in record B and those shown in record C are typical systolic discharges as recorded with intracellular microelectrodes (see also figs. 12 and 13). Soon after the end of record B the microelectrode came out of the cell. Record C was obtained during a new impalement in the same site. Calibrations in A apply also to B and C.

vigorous mechanical activity. A number of measures and devices were tried, with only partial success, to counteract these factors. For instance, several species of anura and specimens of different ages have been employed. Proteolytic enzymes (trypsine and papaine) were used in some experiments in order to digest partially the connective tissue although no obvious difference could be observed. Our best results were obtained by inserting the microelectrodes into the inner surface of the heart after limiting its movements by means of a small glass ring (about 1 mm I.D.) applied with gentle pressure. In spite of this, we have obtained only a rather incomplete picture of the electrical activity of the lymph heart membrane.

If the movement of the heart is not completely restricted and the micropipette tip is advanced slowly towards the inner surface of the heart one can see, when contact with the tissue is first established, that the microelectrode tip goes briefly into the cells on each systole. This is shown by the appearance of short-lasting resting potentials coinciding with the heart contractions (see fig. 11A). The magnitude of these brief potentials goes usually up to 45 or 50 mv, although figures of 60 mv have been observed a few times. Their exact value depends, presumably, upon the extent of the cell damage produced by the erratic insertions. We regard, therefore, the largest value (about 60 mv) as a more faithful representative of the real potential difference across the cell membrane than the average value of the smaller resting potentials usually encountered.

If the microelectrode tip is pushed further into the cell, in an attempt to achieve a more lasting insertion, the resting potential falls usually to a value of between 20 and 30 mv. It stays at this low level during a few systoles before disappearing altogether.

More durable insertions are obtained when the pressure exerted by the glass ring is increased until the movements of the tissue within the ring are fully restrained. Resting potentials of between 40 and 50 mv lasting several systoles are often obtained and, in a few instances, values of above 50 mv could be held for a few seconds. It can be seen, in these records, that each lymph heart systole is a
companied by a burst of potential chang
consisting of several relatively fast co
ponents, or "peaks," which represent me
brane depolarizations, followed by a slow
wave of hyperpolarization (see figs. 11
and 12).

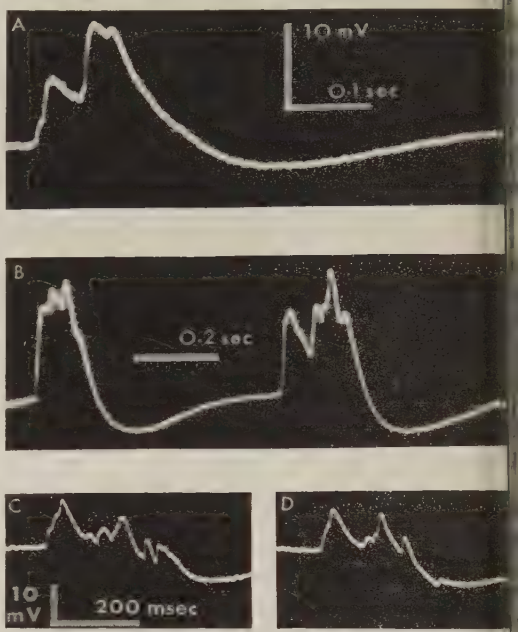


Fig. 12 A, B and C, changes in the potentia
across the surface membrane of lymph hear
muscle cells associated with systolic contraction
The resting potentials on which these potentia
changes were superimposed measured just ove
40 mv. The voltage calibration in record A ap
plies also to B. Calibrations in C apply also to D

The magnitude of the systolic potentia
changes depends mainly upon the value o
the resting potential on which they ar
superimposed. When this is low (20 t
30 mv) the amplitude of the "peaks" i
only 3 to 4 mv. With resting potentials o
40 mv they may reach up to 15 mv. Th
largest systolic depolarizations recorded b
us had an amplitude of just over 30 mv
on a resting potential of 53 mv. In this
instance, single peaks of up to 20 mv in
amplitude could be distinguished.

In spite of the existence of the above
relationship, no attempt has been made
to correlate exactly the amplitude of the
peaks with the resting potential, as their
size also depends upon other factors not

ll understood. Very often the amplitude the peaks, rather large immediately er the insertion, was seen to decay very bidly, well before any appreciable change the value of the resting potential was served. In some instances, it was pos- le to increase the magnitude of the orded resting potential by pushing the croelectrode a little further into the tis- e. This maneuver, however, failed to rease the size of the systolic discharge. disappearance seems to be associated, erefore, with factors other than the es- olishment of a shunt around the micro- ctrode tip, perhaps with the damage oduced to the motor nerve endings. It ust be emphasized, however, that neither e increase in resting potential when the of the microelectrode is pushed fur- er into the tissue, nor the evanescence the systolic potentials was the result an increase in the microelectrode im- dance and associated changes in the equency response characteristics of the cording system.

Each single component of the systolic scharge, as recorded with intracellular ctrodes, consists of a relatively fast ris- g phase followed by a slower decay. The ne of rise to peak of these potentials ries considerably, from 8 to 23 msec., en within a single burst, as can be seen in figure 13. We don't know the reason for those differences although they are probably an expression of the different degree of electrotonic distortion suffered by potentials originating at various distances from the recording micropipette.

Each of the fast depolarizations is followed by a slow decay. Occasionally, the interval between two consecutive fast deflections is relatively long, of up to 60 or more msec. In these instances one can see how the slow repolarization of the membrane is continued smoothly by a phase of hyperpolarization, or increased negativity of the cell interior (see records C and D of fig. 12). One is justified, therefore, in regarding the diphasic change in the membrane potential (i.e., a fast depolarization followed by a more prolonged hyperpolarization) as the basic component of the systolic discharge.

As these components occur usually in rapid succession, what we see in the records is the result of their summation. The fast peaks add up one upon another creating a wave of depolarization of variable amplitude and irregular and spiky contour. This wave is followed by a smooth and continuous wave of hyperpolarization in which the contributions of each single component cannot be distinguished. The behavior of the diphasic changes in mem-

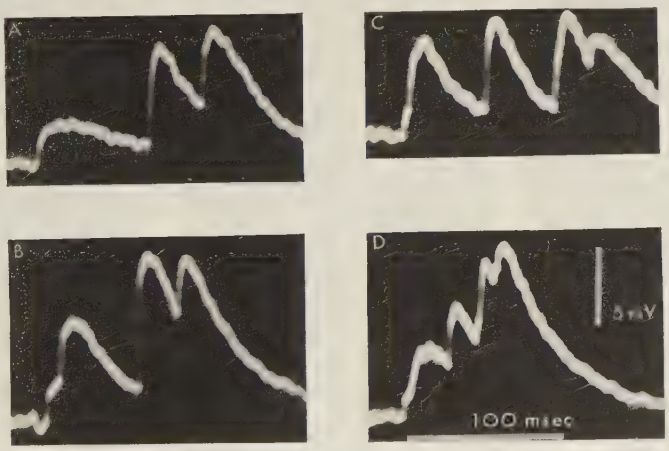


Fig. 13 This figure illustrates the initial phases of 4 systolic discharges of a lymph heart, recorded with a micropipette inserted into a muscle cell. These records were selected because in this cell the initial phase of the discharges was made up of only three or 4 fast components, instead of 5 to 10 as usually found. Notice the large differences in the time course of individual fast waves (see text). Calibrations in record D apply also to A, B and C. The resting potential on which these potential changes were superimposed was of only 35 mv.

brane potential seems to be identical, therefore, with that of the diphasic action currents recorded with external electrodes.

An attempt was made to obtain information on the distribution of the amplitude of the systolic discharge, as recorded with intracellular microelectrodes, over the inner surface of the lymph heart. This proved to be impossible in practice. Although in some preparations sites could be located where potentials of relatively large amplitude were consistently obtained, it could not be ascertained whether the potentials were, in fact, larger there or the conditions for recording more favorable.

Relationship between the systolic discharges recorded with external electrodes and intracellular micropipettes

There is a marked resemblance between the systolic discharges recorded with a single "active" external electrode placed upon the lymph heart and the intracellular potentials just described. In spite of such an obvious similarity (compare, for instance, figs. 2 and 12B) one would like to find out the exact correspondence tween the two events by recording sim taneously with external electrodes a intracellular microtips, from the same s of the lymph heart tissue.

It has been impossible so far, to obser intracellular potentials of the lymph he muscle cells while the external action c rents were recorded with the electro arrangement used in figure 2. Howev in a few exploratory experiments we ha been able to pick up intracellular pote tials at the same time that the action c rents in the vicinity of the microtip we recorded with a couple of thin platinu electrodes separated by a distance of abo 1 mm. These electrodes were connect to a differential amplifier whose outp was displayed in one of the beams of t CRO. The intracellular potentials we shown in the other beam. Figure 14 illu trates some of the records obtained wi this technique. They show that the po tive peaks of the *extracellular* action c rents coincide with the rising phase of t fast *intracellular* potentials (i.e., depola zations) while the external negative d

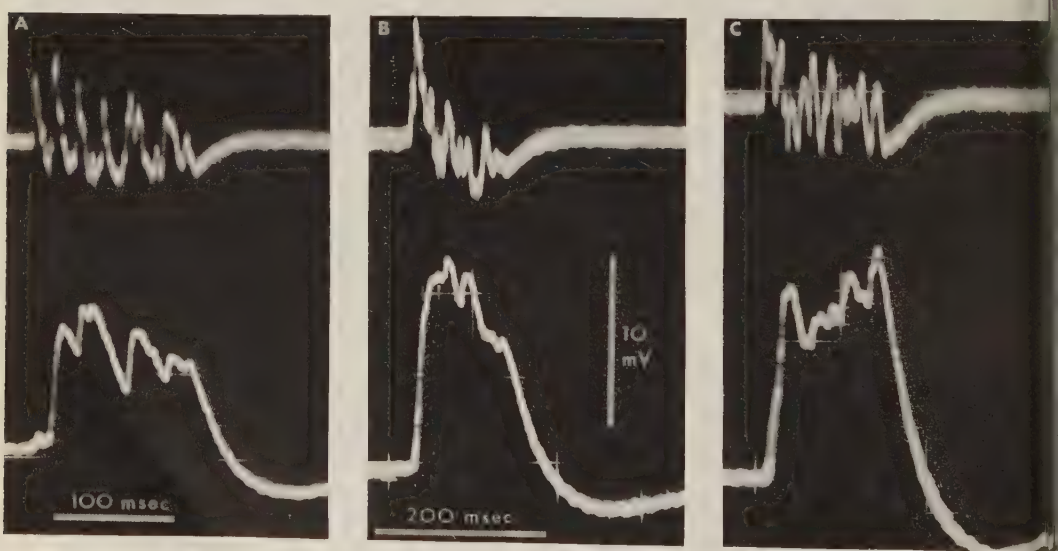


Fig. 14 This figure shows three systolic discharges of a lymph heart recorded simultaneously with a couple of extracellular platinum electrodes (upper trace) and an intracellular micropipette (lower trace). (See text.) The voltage calibration which appears in record B applies also to A and C (intracellular records only). Time calibration in record B applies also to record C. A comparison of the extra and intracellular deflections in this and other records shows that the externally recorded action currents behave approximately like the first derivative of the simultaneous changes in membrane potential.

ions are simultaneous with the re-
rization of the membrane.
lthough better records are needed to
blish accurately the correlation be-
n changes in membrane potential and
on currents, it seems from the present
lts that the extracellular action cur-
s behave approximately like the first
vative of the intracellular potentials.
s finding is consistent with the assump-
that, in most instances, the electrical
vity of the lymph heart recorded with
rnal electrodes is due to current flow-
across areas of membrane that remain
sive during the generation of systolic
harges. The fast positive deflections
erved with external electrodes during
initial phase of the systolic discharge
ld be produced by current flowing out-
ds across the cell surface membrane,
ing the rising phase of the depolariza-
s.
t also appears that the hyperpolariza-
of the membrane, and the subsequent
rn of the membrane potential to the
ial level, occur at a rate too slow to be
ected in an external action current
e enough to be detected with the ex-
imental arrangement used to obtain
records of figure 14. However, a care-
examination of these records shows
t the final and slower, negative deflec-
that marks the end of the extracellular
tolic discharge does not end as soon as
membrane potential reaches the di-
olic level, but goes on slightly beyond
t point. This short tail may be re-
ded as a very attenuated extracellular
nterpart of the phase of membrane
perpolarization.

DISCUSSION

The experiments described above show
t the systolic discharges of the lymph
art are produced by the addition of a
mber of diphasic variations of the rest-
g potential existing across the surface
embrane of the lymph-cardiac muscle
ls. Each of these potential changes,
cited by volleys of motor nerve impulses,
nsists of a relatively fast depolarization,
up to 20 mv in amplitude, followed by
slower phase of hyperpolarization.
The first question raised by the demon-
ration of the diphasic lymph heart po-
tentials is that of their nature. In principle, each potential could represent a post-synaptic potential, an action potential, or a combination of both. Their time course does not provide much information on this problem. Although the duration of each of the components of the systolic discharge is long compared with the action potentials recorded from nerve and skeletal muscle of Amphibia, it is short compared with the action potentials recorded from the frog's ventricle (up to 0.8 sec. at 21°C, according to Woodbury et al., '51) or the frog's sinus (with an overall duration of up to 1.6 seconds, at 16°C, del Castillo and Katz, '55). An action potential, however, represents an all-or-nothing event in which the potential difference across the excitable membrane reaches a fixed level. For this reason, action potentials cannot be added in single cells. The intracellular lymph heart potentials, however, add one upon another, suggesting that we are dealing with post-synaptic potentials rather than with action potentials.

Another indication of the synaptic nature of those potentials is the effect of d-tubocurarine on the lymph heart action currents. As figure 10 shows, the establishment of a curarine block is a gradual process not accompanied by noticeable changes in the patterns formed by the action currents.

The experiments in which the influence of dtC, ACh and atropine were studied strongly suggest that the neural activation of the lymph heart depends upon a synaptic mechanism involving the liberation of ACh and its combination with postsynaptic receptors. It is still doubtful whether an enzymatic ACh-inactivating mechanism operates in the neuromuscular junctions of the lymph heart. The results of experiments in which prostigmine at concentrations of 2 to 4×10^{-6} w/v were applied to the lymph heart have so far been negative. It is likely, however, that more refined techniques may be required to demonstrate the effects of anticholinesterasic agents.

Potentials resembling the miniature end-plate potentials recorded at the skeletal neuromuscular junctions have occasionally been observed. However, the

uncertainties associated with the measurement of the actual amplitude of the synaptic potentials, emphasized earlier made it difficult to decide whether those potentials were due to spontaneous transmitter liberation or to uncoordinated firing of single spinal motoneurones. Erratic discharges of the spinal lymph heart centers can be observed while asphyxia of the central nervous system develops. When this happens the activity of the lymph heart becomes disorganized before ceasing altogether. The vigorous systolic contractions of the heart are replaced by a weak "fibrilation." Intracellular recording shows then the occurrence of single diphasic potentials not grouped into systolic discharges.

In experiments such as the one illustrated in figure 8A, it can be observed that the time course of single action currents elicited during the phase of hyperpolarization following a previous current is different from those elicited in a resting membrane. Experiments are in progress to determine whether this difference is the consequence of a mechanical artifact or due to a change in the properties of the membrane of the lymph-cardiac muscle cells during the hyperpolarization.

As no action potentials have been observed, it appears that the activation of the contractile machinery of the lymph-cardiac cells is elicited directly by synaptic activity.

Two different aspects should be considered separately when summarizing the above results. In the first place, some of the functional properties of the lymph heart depend upon the patterns of discharge of the spinal centers that supply motor innervation to this organ. Our results in this regard have confirmed earlier work. The records obtained from bundles of fibers of the XI spinal nerve have shown that each lymph heart systole is elicited by a train of motor impulses made up by several (5 to 10) separate volleys. It is likely that each motor axon may contribute one or more impulses to each volley, although the number of action potentials and the duration of each volley, as well as the sequence of fiber discharge, are highly irregular and seem to vary at random from systole to systole.

In the second place, we should co sider the intrinsical properties of t lymph heart muscle cells. These are ch acterized, from an electrophysiologi viewpoint, by (a) a resting potential wh amounts roughly to ⅔ of that of skele muscle fibers, (i.e. about 60 mv, insi negative), (b) the absence of action tentials, and (3) the occurrence of phasic synaptic potentials, in which t depolarization of the cell membrane up to 20 mv) elicited by a choliner mechanism is followed by a longer pha of membrane hyperpolarization. The three properties, together with a diffu and relatively uniform distribution nerve endings, are the distinctive featur of the slow muscle system of the Amphib studied by Kuffler and Vaughan Willian ('53; see also Kuffler, '53). Althou more work is needed to reach a defin conclusion we may tentatively regard t lymph heart as an organ made of "slo muscle fibers. We have already mention the difficulties encountered when trying study with electrical methods the patter of distribution of motor nerve termina in the lymph heart. It is likely that info mation on this problem could be obtain more easily from histological studies.

The systolic discharges recorded fro the spontaneously beating lymph heart a the result of the summation of a numb of diphasic synaptic potentials, each eli ited by a separate volley of nerve impulse Due to the great variability of those vo leys with regard to the numbers of in pulses, overall duration and sequence firing of spinal motoneurones the shap duration and distribution of the systoli action currents are highly changeab affairs.

SUMMARY

1. The electrical activity of the post rior lymph hearts of the toad has been in vestigated using both external electrod and intracellular micropipettes.

2. In agreement with previous wor bursts of muscle action currents (systoli discharges) were found to be associate with each lymph heart systole. Mechanica activity starts about 20–25 msec. aft the beginning of each burst, reaching maximum at the end of it.

. If one external electrode is in con- with the dorsal wall of the lymph rt and the other is placed upon the rounding connective tissue, the first of the systolic discharge appears as arge serrated deflection made of the n of several (5 to 10) relatively fast itive waves. This is followed by a oth and uninterrupted negative deflec-

. If the lymph cardiac motor axons are nulated with a single electric pulse, a hasic action current is recorded from muscle tissue. Repetitive stimulation ws that the systolic discharge described (3) is the sum of a number of these hasic currents.

. The electrical activity of lymph car- motor axons has been recorded simul- eously with the systolic discharges. h muscle action current is seen to be ceded by a volley of motor impulses.

. The impalement of lymph heart mus- cells with an intracellular micropipette eals the existence of a resting potential p to 60 mv in amplitude (cell interior ative).

. The systolic discharges recorded h an intracellular microelectrode begin h a number of relatively fast deflections t form a spiky wave of depolarization p to 30 mv in amplitude. This phase ollowed by a wave of membrane hyper- arization of smooth and continuous tour.

. The intracellular systolic discharge, same as its extracellular counterpart, he result of the addition of a number diphasic deflections, i.e., fast depolari- ions followed by more prolonged hyper- arizations. These potential changes may regarded as postsynaptic potentials pro- ed by a cholinergic mechanism. No ion potentials have been recorded from lymph heart.

. The electrical activity of the lymph rt has been recorded simultaneously h an intracellular pipette and a couple extracellular electrodes placed in the inity of the microtip. The external ac- n currents recorded with this electrode angement behave approximately as the t derivative of the changes in mem- ne potentials.

10. It appears from the above results that the electrical activity of the lymph heart muscle resembles very closely that of the slow muscle system of the Amphibia.

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e Possible Role of DNA-ase I in DNA Replication

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t is a well-established fact that native o-stranded DNA which has been isolated m calf thymus cannot itself act as a imer" unless it has been denatured (usu- by heat) (Bollum, '59), in the syn- sis *in vitro* of DNA from the 4 deoxyri- nucleoside triphosphates using the poly- rizing enzyme from thymus tissue. This o-strand DNA does function as a primer wever in Kornberg's system using an zyme prepared from *E. coli* (Lehman et '58). In the latter case a slight degra- tion of the DNA with DNA-ase increases rate of reaction two or three-fold (Leh- n, '59).

When using an enzyme obtained from erphase liver cells, it has been found t unheated DNA does not act as a mer (Mantsavinos and Canellakis, (a), but when the enzyme is obtained m regenerating liver, two-strand DNA l act as a primer. In the latter case the dition of DNA-ase I decreases the nount of synthesis at all concentrations vestigated. When polymerizing enzyme m certain tumor cells is used, an opti- l concentration of DNA-ase I enhances e net synthesis 5-fold (Mantsavinos and nellakis, '59b). It is thus obvious that e rate of synthesis of DNA from the oxyribonucleotide triphosphates can be fluenced either positively or negatively DNA-ase action.

Consideration of the results just out- ed leads to the suggestion that native o-stranded DNA might need to be con- rted to single strand material prior to NA synthesis. This idea is compatible th the fact that the single-stranded DNA hich has been isolated from Φ X174 age by Sinsheimer ('59) can act as a imer for the polymerizing enzyme from ymus (Bollum, '59) or from *E. coli* (Leh- an et al., '58). The idea is also compati- e with recent work of Zamenhof ('59) indicating an instability of one of the strands of the two-strand DNA of *E. coli*. However, the question as to whether complete separation of the two strands ("de-stranding") is necessary for primer action has not yet been settled.

It appears somewhat difficult to imagine a process that could produce material *in vivo* similar to the heated DNA. The idea of completely unwinding the two strands of a double helix prior to DNA synthesis under physiological conditions would seem to present rather formidable difficulties (Delbruck and Stent, '57), although such unwinding or "destranding" may well occur on heating DNA. Hence it seems worthwhile to consider the possibility that single-stranded DNA might be produced as the result of a specific destruction of one strand of the two-stranded helix. It should be noted that either strand of a two-stranded Watson-Crick helix carries all the information needed for the synthesis of more two-stranded DNA.

If destruction of one strand of a DNA double helix were to precede DNA synthesis, the first step in the synthetic process would presumably have to be the exact duplication of this single strand material. Then the single strands could conceivably bind the complementary bases to themselves according to the A-T, G-C rule, and finally after polymerization of the newly bound bases, two double strands of DNA would have been formed for each double strand originally present. This second step in the synthesis could terminate the process according to the suggestion of Bollum ('59), if some way could be found to block further conversion of double to single strand material. A mechanism of this sort might or might not be compatible with the

[1] We gratefully acknowledge the support of the National Cancer Institute, U. S. Public Health Service grant C-994(C10).

results of Meselson et al. ('57) on isotope distribution in DNA after replication.

Since DNA-ase I is capable of enhancing the primer activity of two-strand DNA in certain cases, as stated above, it seemed logical to determine whether DNA-ase I action on the nucleoprotein complex of cell nuclei might lead to the production of single-strand DNA. The reasoning behind this proposition is that one of the fibers of the two-stranded DNA in the nucleoprotein might be sterically hindered by protein, especially by histone, and thus be inaccessible to DNA-ase action.

It was considered of interest to investigate this possibility by studying the action of DNA-ase on cell nuclei isolated at pH 6.0 using very dilute citric acid as a medium. In this case DNA-ase I is liberated from cytoplasmic granules and enters the nuclei during the homogenization and afterwards, causing degradation of the DNA (Dounce et al., '57). We have found that a mild but definite degradation of the DNA occurs if the pH is kept between 5.8 and 6.0, whereas a more extensive degradation takes place if the pH is allowed to rise to 6.1 or 6.15. Above pH 6.2 the nuclei are disintegrated. The two types of DNA in question will subsequently be referred to as *slightly degraded DNA* and *considerably degraded DNA*, respectively. The viscosity of the *slightly degraded* DNA has already been reported (Dounce et al., '57), while that of the *considerably degraded* DNA was about 1/5th that of calf thymus DNA in 0.01 M NaCl solution.

The immediate object of our investigation was to isolate DNA from the already isolated liver cell nuclei and then determine whether the degradation was entirely the result of simple depolymerization (i.e., transection of double helices) or whether a tendency towards production of single stranded material might be noted. We have elected to use the action of DNA-ase I in the homogenate on the whole nuclei rather than to study the action of crystallized DNA-ase I on isolated nucleohistone in order to keep conditions as close to physiological as possible. Isolated DNA nucleohistone may represent a more or less disrupted structure, the study of which might in this case be more apt to lead to erroneous conclusions than the use of the more complicated system involving the wh nuclei where the DNA-ase action on DNA takes place as it were *in situ*. know that histones remain in the pH nuclei (Monty and Dounce, '57) and know that the chief enzyme degrading DNA is DNA-ase I (Dounce et al., '57; also their references).

In order to test for the production single strand material, the formaldehy reaction of Fraenkel-Conrat ('54) was e ployed, using spectral shift as a measu of the reaction. In addition, studies of ba ratios of the isolated DNA were made the method of Wyatt ('55).

Studies of base ratios of course will n show whether there has been separation the two strands of the DNA double hel provided that both strands remain in t sample. If, however, there has been part destruction and loss of one of the t strands, analysis for base ratios would expected to show some variance from t A-T, G-C rule.

For purposes of comparison, some stu ies were also made of calf thymus DN DNA isolated from whole rat liver, a DNA prepared from rat liver cell nucl previously isolated at pH 4.0 (where t DNA is not subject to enzymatic degrad tion). In addition studies were made calf thymus DNA that had been degrad by means of the Waring blendor. In a cases where the formaldehyde reaction w used, the effect of heat in enhancing t reaction was studied. Since dissolving distilled water below pH 7.0 tends to di rupt the DNA double helix, resulting in t development of a considerable reactio with formaldehyde, the DNA was generall dissolved in 0.01 M NaCl prior to testin The effect of distilled water at pH 7.0 thereabouts was also studied in some case as well as the effect of distilled water at pH of about 8.5. In the latter case t double helix may be even more stable tha it is in 0.01 M NaCl.

EXPERIMENTAL DETAILS

Cell nuclei were isolated from rat live at pH 5.8 to 6.0 by the method describe by Dounce ('55). These nuclei containe what we have termed *slightly degrade* DNA. The degradation results from th action of cytoplasmic DNA-ase which i

ased from cytoplasmic particles during homogenization. The method also was dified by holding the pH between 6.0 l 6.15 with the purpose of obtaining at we have termed *degraded* DNA. In s case the method for isolating the nu- is more difficult to carry out and the d is low, owing to the disruption of re of the nuclei than occurs when the is held between 5.8 and 6.0. DNA was ated from the above two types of nuclei the method of Kay et al. ('52).

DNA was also isolated from calf thymus the same method (Kay et al., '52), and mples of this material were subjected to radation by the action of a Waring ndor. In these experiments 100 mg of DNA was dissolved in 200 ml of 0.01 NaCl and then was allowed to remain three minutes in a Waring blendor run- g at full speed. After this the DNA was recipitated by adjusting the NaCl con- tration to 1 M and adding one volume ethanol. The reprecipitated material s washed twice with 75% ethanol, twice h 95% ethanol, and finally was dried h ethyl ether.

DNA-ase I-degraded DNA was obtained reating a 100 mg sample of calf thymus A dissolved in 100 ml of 0.01 N NaCl ution with 2.5 mg crystalline DNA-ase orthington) for 5 minutes in the pres- e of 0.002 M Mg-acetate. The degraded A was isolated from the solution by add- an equal volume of 95% alcohol in the sence of 1 M NaCl. The precipitated A was washed twice with 75% alcohol, l twice with 95% alcohol, and finally s dried with ether.

The formaldehyde reaction was carried according to the directions of Fraenkel- nrat ('54).

RESULTS AND DISCUSSION

We have found that the only DNA sam- s showing any evidence of the presence single strand material before heating, as ged by the formaldehyde reaction, were *degraded* DNA samples and the calf mus DNA degraded by means of the ring blendor. This finding correlates h the primer activities of the samples, ich were measured by Dr. F. J. Bollum,[2] ce only the *degraded* liver DNA (de- ded by DNA-ase *in situ*) and the Waring

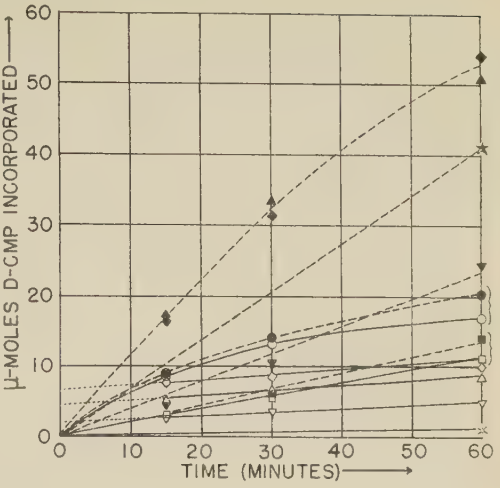


Fig. 1 Priming activities of various types of DNA described in text, before and after heating. ○, ●, *Degraded* DNA (from rat liver nuclei) after treatment with 6 M urea. □, ■, *Waring blendor-degraded* DNA. △, ▲, *Degraded* DNA (from rat liver nuclei). ◊, ◆, *Partially degraded* DNA (from rat liver nuclei). ▽, ▼, DNA extracted from rat liver nuclei isolated at pH 4.0. ⚹, ★, Calf thymus DNA. Solid line with open symbols indicates priming activity before heating and dotted line with solid symbols refer to values obtained after heating. All heated samples were heated at 100°C for 10 minutes. Data for all curves obtained from equal aliquots of the same DNA solution. (This is also true for all subsequent figures.)

blendor-degraded calf thymus DNA showed appreciable primer activity before heating (see fig. 1).

We have subjected all DNA samples to analysis of base ratios according to the method of Wyatt ('55), as well as to the formaldehyde reaction before and after heating. The results of analyses for base ratios are shown in table 1. The base ratios of the enzyme-degraded samples (*slightly degraded* and *degraded*) are not normal for intact DNA, and this taken by itself indicates a certain tendency towards single strand formation. However it was necessary to treat the DNA samples with 0.3 N KOH at 37°C for 18 hours before carrying out determinations of base ratios, since all DNA samples isolated from pH 4 liver cell nuclei were contaminated with RNA to

[2] We are greatly indebted to Dr. F. J. Bollum of the Oak Ridge National Laboratories for carrying out a number of tests of DNA samples for primer activity in his system for DNA synthesis using thymus enzyme (Bollum, '59).

TABLE 1

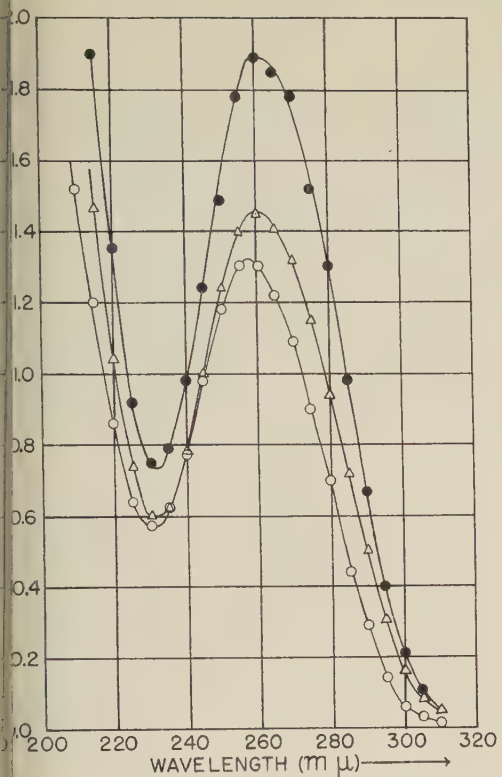
	DNA sample[1]	A	T	G	C	A/T	G/C	PU/Py	$\frac{A+T}{G+C}$	% RNA before alkali treatment
1.	Calf thymus	1.00	1.04	0.75	0.75	0.95	1.00	0.97	1.35	3.8
2.	Whole rat liver	1.00	1.04	0.73	0.61	0.96	1.19	1.04	1.51	8.2
3.	pH 6.0 nuclei, partly degraded	1.00	1.34	0.87	0.78	0.74	1.11	0.88	1.41	42
4.	pH 6.0 nuclei degraded	1.00	1.26	0.81	0.89	0.79	0.91	0.84	1.32	33
5.	pH 6.0 nuclei partly degraded, urea-treated	1.00	0.95	0.71	0.59	1.05	1.20	1.11	1.48	21
6.	pH 6.0 nuclei, degraded, urea-treated	1.00	0.93	0.76	0.60	1.06	1.25	1.14	1.32	31
7.	pH 4.0 rat liver nuclei, DNA isolated at pH 6.0 soluble RNA first removed by extraction with 0.9% NaCl	1.00	1.04	0.73	0.65	0.96	1.11	1.02	1.46	31
8.	Rat liver nuclei isolated at pH 4.0, DNA isolated from them at pH 6.0. Soluble RNA not removed first	1.00	0.79	0.56	0.61	1.25	0.92	1.11	1.51	48
9.	Rat liver nuclei isolated at pH 4.0, DNA isolated from them at pH 7.0 soluble RNA not removed first	1.00	0.76	0.42	0.61	1.29	0.74	1.06	1.76	45

[1] All samples treated with 0.3 N KOH at 37°C for 18 hours before analysis—DNA recovered by precipitation with perchloric acid. Each figure in the table represents the average of duplicate analyses.

the extent of 30 to 40% (see table 1), and even the calf thymus DNA contained 3 to 4% of RNA.[3] As the *slightly degraded* DNA did not show a reaction with formaldehyde and hence did not exist to an appreciable extent in the form of single strand material, it is likely that any single strand formation indicated by analysis of base ratios was brought about by the action of the alkali in dissolving away degraded single strand fragments from more intact complementary chains.

The extent of reaction of the *degraded* DNA samples with formaldehyde before and after adding urea is shown in figures 2, 3, and 4. Control experiments with undegraded calf thymus DNA are illustrated in figure 5. A tendency towards the formation of single strands is indicated for the *degraded* DNA by the results of th

[3] We now know that this difficulty could have been largely avoided by precipitating the DNA in all cases with ½ volume of ethanol instead of with one volume from the saline detergent solution. However RNA does not have primer activity in Bollum's system and moreover treatment with alkali is a standard method to remove RNA before doing analysis for base ratios on DNA, so that as far as we can see the presence of RNA in the DNA samples does not affect our conclusions in any important manner.



ig. 2 U.V. spectrum of *degraded* DNA ex-ted from rat liver nuclei isolated at pH 6.0–5, dissolved in 0.01 M NaCl. ○, Degraded A, no formaldehyde, no heating. △, Degraded A after reaction with formaldehyde, no heat-. ●, Degraded DNA, after heating at 100°C for minutes, formaldehyde added after cooling so-ion. All samples dissolved in 0.01 M NaCl. ating carried out for 10 minutes at 100°C. Con-tration of formaldehyde where used approx. %. (Increase in formaldehyde concentration at trolled pH does not cause further change in ctrum.)

maldehyde reaction, primer activity, and analysis of base ratios.

This urea-treated, *degraded* DNA is of nsiderable interest. It was originally ped that urea treatment would cause the moval of small oligonucleotide single ains from their more intact complemen-y neighbor strands, as suggested above explain the action of alkali on the *de-ded* and *slightly degraded* samples. wever the table of analysis of base ratios ble 1) shows that this probably is not e case, and we believe from the results the following experiments that the main tion of the urea was to facilitate the re-moval of contaminating RNA from the degraded DNA sample.

RNA isolated from yeast by Kirby's ('56) method was separated into two fractions by dissolving in 1 M NaCl and chilling to zero for several hours. A part of the material precipitated (the "less soluble fraction") and this was centrifuged off, leaving a supernatant solution from which a "soluble" RNA fraction was isolated by precipitations with two volumes of ethanol at 0.3°C.

Both the "less-soluble" and "soluble" yeast RNA fractions just described could be largely precipitated from 1 M NaCl solution at a concentration of 1 mg of RNA per ml if one volume of ethanol was added, but in the presence of 6 M urea the "soluble" fraction would not thus precipitate. Six molar urea did not however prevent the

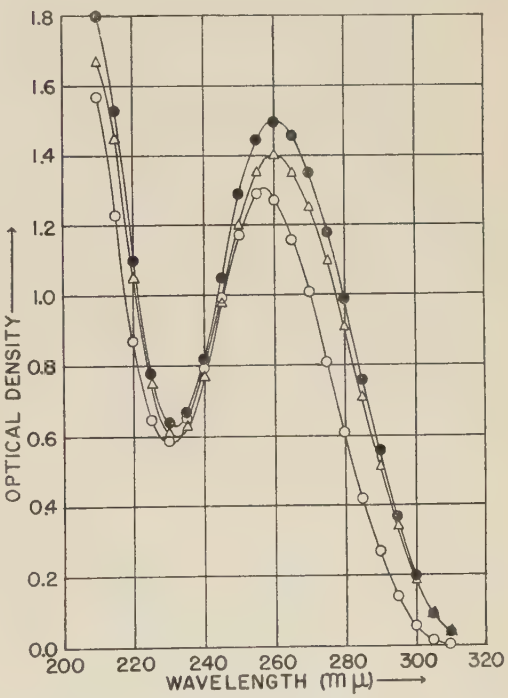


Fig. 3 U.V. spectrum of *urea-treated degraded* DNA extracted from rat liver nuclei isolated at pH 6.0–6.15. ○, Urea-treated *degraded* DNA. △, Urea-treated *degraded* DNA after reaction with formaldehyde, no heating. ●, Urea-treated *degraded* DNA after heating, formaldehyde added after cooling. All samples dissolved in 0.01 M NaCl. Heating carried out for 10 minutes at 100°C. Concentration of formaldehyde where used approx. 1.3%.

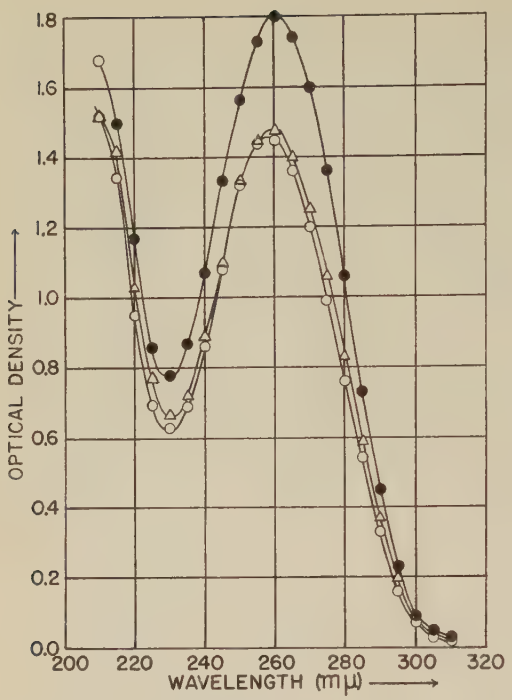


Fig. 4 U.V. spectrum of *urea-treated degraded* DNA extracted from rat liver nuclei isolated at pH 6.0–6.15. ○, Urea-treated, *degraded* DNA. △, Urea-treated *degraded* DNA after reaction with formaldehyde, no heating. ●, Urea-treated *degraded* DNA after heating, formaldehyde added after cooling. All samples dissolved in distilled water at pH 8.5; heated sample held at 100°C for 10 minutes. Concentration of formaldehyde where used approx. 1.3%.

precipitation of the "less-soluble" RNA fraction by one volume of alcohol under the same conditions used for the "soluble" fraction. It is thus clear that if much or most of the nuclear RNA is similar to the "soluble" fraction of yeast RNA isolated by Kirby's method, 6 M urea would be expected to aid in its removal from the DNA during the reisolation procedure. That this is actually the case was confirmed from the results of analysis for ratios of uracil to thymine which indicated that less RNA was present in the *degraded* urea-treated samples than in the *degraded* samples that had not been treated with urea.

It will be noted from table 1 that the results of analysis for base ratios of the *degraded*, urea-treated DNA show that after the urea treatment, the base ratios tend to approach the values for undergraded DNA, although they still are not normal. W attribute this finding to the removal by th treatment with urea of the most degrade fragments of the DNA, leaving less d graded DNA which is more nearly norm in base ratios.

It should be noted that urea does n produce any effect on the *slightly degrade* DNA except to shift the base ratios som what towards those for undegraded DN nor does it have any effect at all, as far a is known, on intact calf thymus DNA. may be that in the case of the *less-degrade* DNA, the contaminating RNA co-precip tates more extensively with the DNA in th presence of 6 M urea, because the DNA still somewhat fibrous.

In regard to the action of DNA-ase I o purified calf thymus DNA in the presenc of 0.002 M $MgAc_2$, it was found that onl about 20% as much enzyme was require to produce a given amount of degradatio as is required to degrade DNA-nucleopr tein *in situ* (Dounce et al., '57). Howeve studies of the calf thymus DNA degrade by crystallized DNA-ase showed result that were practically indistinguishabl from the results of studies of the degrade

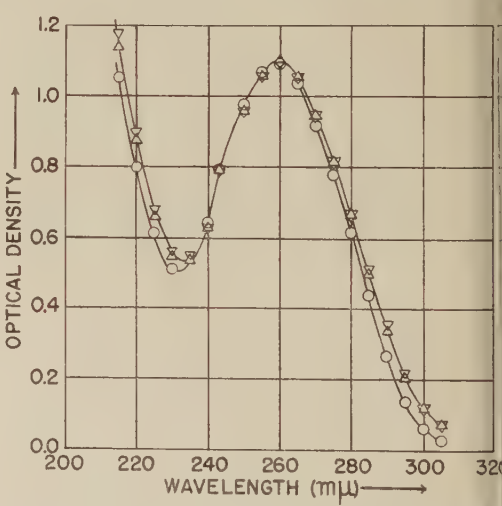


Fig. 5 U.V. spectrum of calf thymus DNA di solved in 0.01 M NaCl under various condition ○, Calf thymus DNA. △, Calf thymus DNA afte reaction with formaldehyde, no heating. ▽, Ca thymus DNA in 0.01 M NaCl, pH adjusted to 5. after reaction with formaldehyde, no heatin Samples dissolved in 0.01 M NaCl in all case Concentration of formaldehyde where use approx. 1.3%.

A from pH 6 liver cell nuclei, the only erence being that the formaldehyde ction was slightly greater in the case of *degraded* DNA isolated from the nuclei. se experiments show that DNA-ase I a certain tendency to attack each nd of the DNA double helix to a differ- extent, even when purified DNA in- d of nucleoprotein is used as substrate, ting to the production of a certain unt of single strand material after tment with alkali; but the presence of one and residual protein seems to exert a non-specific hindrance to DNA-ase on rather than a specific effect on one nd only of the double helix.

he Waring blendor-treated material is of considerable interest. This has dently been converted in part to a de- ured product in which there has been reciable hydrogen bond rupture; but ddition scission of the double helix to n shorter segments also must have oc- red. This is indicated by the fact that bH 8.5, the Waring blendor-degraded lples do not show a reaction with for- dehyde, indicating a reversal of the aturation process, but in spite of this original fibrous nature of the material ot restored when the DNA is precipi- d at pH 8.5 by one volume of alcohol in presence of 1.0 M NaCl.

he effect of formaldehyde on the War- blendor-degraded DNA is shown in fig- 6. The results clearly indicate a mix- of double-strand and single-strand erial.

is of interest that heat treatment has apparent effect on the Waring blendor- raded calf thymus DNA, than on native A. We interpret this to indicate that the denaturation, which certainly must ar, is more reversible in the cases of e degraded samples, at least as judged he formaldehyde reaction, than is the t denaturation of native DNA.

ne other point that requires comment e difference in behavior towards heat he *degraded* DNA (degraded by DNA- *in situ*) and the Waring blendor de- ded-DNA (see figs. 2 and 6). It is pos- e that the increase in primer activity . 1) and in reactivity towards formalde- e of the *degraded* DNA may be caused he presence of RNA in the latter which

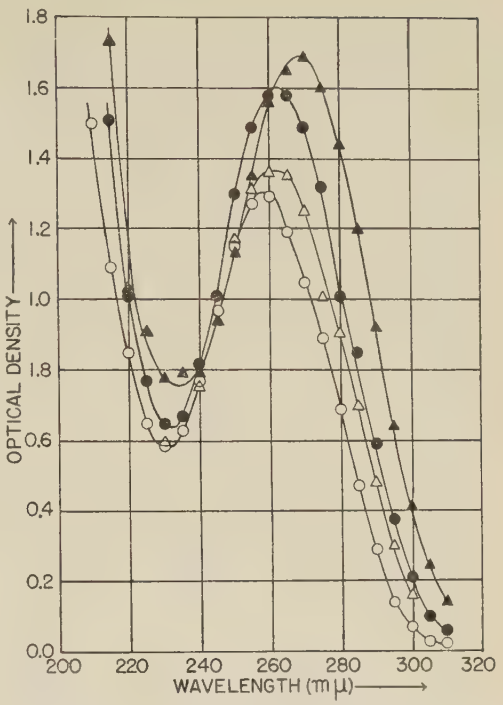


Fig. 6 U.V. spectrum of Waring blendor-degraded DNA in 0.01 M NaCl, after reaction with formaldehyde under various conditions. ○, Waring blendor-degraded DNA. △, Waring blendor-degraded DNA after reaction with formaldehyde, no heating. ●, Waring blendor-degraded DNA after heating for 10 minutes at 100°C, formaldehyde added after cooling. ▲, Waring blendor-degraded DNA after heating, formaldehyde added before cooling solution. Heat-treated sample held at 100°C for 10 minutes. All samples dissolved in 0.01 M NaCl. Concentration of formaldehyde where used approx. 1.3%.

prevents reversal of the heat denaturation on cooling.

GENERAL DISCUSSION

It appears that the action of DNA-ase I on DNA does have a certain tendency to produce more cleavage in one of the two strands of the DNA double helix than in the other. However little difference was noted between the action of the DNA-ase on purified calf thymus DNA and on DNA-nucleoprotein of rat liver *in situ*, and hence the hypothesis that histone might sterically hinder the action of DNA-ase I on one of the two strands of the double helix, does not seem to be valid. Moreover we have not been able to show that single strand cleavage from DNA-ase I action occurs without

concomitant double strand scission. Hence the hypothesis that single strand formation might be brought about by DNA-ase I action prior to DNA replication is not favored by our results. It is true that we have not been able to work under completely physiological conditions but deviations from the latter would not seem to invalidate these conclusions, as far as we can see at the present time.

At least one other enzyme that might conceivably cause single strand formation from double strand DNA is phosphodiesterase, which is an exo-enzyme. However Bollum found no DNA synthesis in his system using thymus enzyme in the presence of phosphodiesterase with double strand DNA (Bollum, '60), although the addition of DNA-ase I to the same system in place of the phosphodiesterase did permit DNA synthesis to occur.

We believe that the reason for the latter observation is explained by the work reported in this paper. Our results would seem to indicate that the DNA synthesis which occurs *in vitro* after action of DNA-ase on the primer DNA probably does not represent a process that takes place under physiological conditions *in vivo*.

SUMMARY

1. The action of DNA-ase I on the nucleoprotein complex of the cell nucleus was investigated by allowing DNA-ase I of rat liver, liberated from cytoplasmic particles during homogenization of the liver, to act on cell nuclei present in the homogenate. A *degraded* and a *partially degraded* DNA was isolated from nuclei which had been isolated from the homogenates at pH 6.0 to 6.15 and from 5.8 to 6.0 respectively in the presence of very dilute citric acid.

2. The *degraded* DNA showed some activity in Bollum's system for DNA synthesis by thymus enzyme, and also showed some reactivity with formaldehyde without being heated, and on analysis showed abnormal base ratios. These results indicate a tendency toward single strand production, although concomitant double strand scission also occurs. The same was true for DNA degraded by action of a Waring blendor.

3. Purified calf thymus DNA treated with crystallized DNA-ase I behaved similarly to the degraded DNA isolated fr the rat liver nuclei. Hence it can be c cluded that the action of DNA-ase on D *in situ* during isolation of the nuclei is affected qualitatively in an important w by the presence of protein in the nu (histone or residual protein).

Quantitatively, the reaction appears be considerably slower in the presence these proteins.

4. Treatment of the degraded DNA fr the nuclei with 6 M urea did not appear increase the amount of single-strand ma rial present but probably facilitates the moval of contaminating RNA.

5. It is concluded from the results giv above that formation of single-strand D by an attack of DNA-ase I on one of t two strands of double helix DNA prior DNA replication *in vivo* probably does n occur.

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rystalline Luciferin from Live *Cypridina*[1,2]

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The small ostracod crustacean, *Cypri-a*, produces a brilliantly luminescent retion wherein light emission accom-nies the aerobic oxidation of a substrate, iferin, catalyzed by an enzyme, lucifer-e. Crystallization of the luciferin, ex-cted from whole, dried organisms, was hieved in 1957 by Shimomura, Goto and rata, partly with the aid of purification ocedures developed by Harvey and his sociates through many years of research this system, (cf. Harvey, '52; Tsuji, ase and Harvey, '55).

A new approach in the efforts to isolate re luciferin, involving the use of living her than dried organisms, was inde-ndently undertaken in 1957 by Johnson d Sie, with the cooperation of Haneda, katsuki, Sugiyama and Masuda in Shi-oda. This approach was based on the emise that the luminescent secretion tained from living organisms by elec-ical stimulation or other means should relatively free of many diffusible sub-ances contained in whole organisms, es-cially after degradation reactions that doubt occur during the process of slow ying, usually accomplished by exposure the sun. With living organisms, paper romatograms of the luminescent secre-on, drawn into methyl alcohol in a suc-on flask immediately after stimulation, nded to justify the original premise, but rious circumstances made it difficult to tain an amount of secretion sufficient r the purposes in view. On the other and, extracts of initially living, whole ganisms appeared to offer certain ad-antages over those of dried, whole organ-ms, e.g., in having a smaller number impurities and a larger content of luci-rin.

The present report is based on the further pursuit of this approach, which has led to a considerably simplified procedure for the isolation of luciferin and to its crystallization in appreciable quantities for the first time from summer organisms, whose luciferin has been found to be less in amount and more difficult to purify than that of organisms collected during the colder seasons of the year.

EXPERIMENTAL METHODS AND RESULTS

Warm methanol extracts

Anderson ('35) noted that, although the solubility of luciferin in different solvents varied widely with biochemical treatment of the material (starting with dried organisms), the active substance was spectacularly soluble in methanol under all conditions. A number of methanol extracts were prepared in 1957 at Shimoda, by plunging the living organisms into a large volume of methanol in a suction flask at room temperature, evacuating the air, shaking vigorously, sometimes in a warm water bath, then filtering through paper in a Buchner. The filtrate was always rich in yellow or greenish yellow substances, and highly fluorescent with a somewhat similar color in ultraviolet. Repeated extraction increased the yield of such substances, indicative of luciferin, and highly

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active in light emission with luciferase. These filtrates were subjected to various treatments before ultimately drying and sealing in vacuo for further fractionation in the Princeton laboratories. The light-emitting potency of the residues was found to vary with the previous treatment and with unknown factors, when tested later by means of a photomultiplier-light integrating apparatus. The best preparations had a potency of some 50,000 L.U. ("Light Units," arbitrarily defined units of total light emitted with luciferase) per milligram, whereas the ground powder of whole, dried organisms was often not more than 50 L.U./mg and did not exceed 300 L.U./mg in the most active of such material available.

The most nearly pure luciferin obtained from these preparations was by the following procedure. A net weight of 193 gm of living organisms was added to 600 ml methanol, the air evacuated, and the flask heated in a water bath at 65°C for one hour. The solution was cooled, filtered, and the methanol distilled off in vacuo, leaving about 70 ml of aqueous solution which was then extracted with benzene in a separatory funnel containing an argon atmosphere to retard the decomposition of the very oxygen-labile luciferin. The reddish benzene fraction, containing reddish solids at the interphase, exhibited a strong light-emitting activity with luciferase, but the yellowish aqueous fraction had much stronger activity. The latter was extracted with butanol, removing most of the luciferin, and the solvent was removed by vacuum distillation in a water bath at 80 to 90°C. The residue was taken up in methanol, transferred to an ampoule, the methanol then removed in vacuo, and the ampoule sealed.

Several weeks later the dry residue was found to have a light-emitting potency of 25,000 L.U./mg. It was then extracted with benzene in a Soxhlet, followed by dissolving in a small volume of Tsuji's ethanol-ethyl acetate-water solvent (Tsuji, '55) and placing on a cellulose column that had been deaerated by hydrogen purified over hot platinized asbestos. The column was developed in a hydrogen atmosphere by Tsuji's solvent that had been deaerated with hydrogen. Although 5 bands were visibly evident on an essentially similar column of material from dried organisms, after purification by prolonged extraction with benzene and ether followed by two cycles of a modified Anderson's ('36) benzoylation procedure (Shimomura et al., '57) only 3 bands were evident in the present instance. They were, (1) an orange colored, non-fluorescent band at the front, followed by a yellow, yellow fluorescent (in ultraviolet) band, and finally a colorless, blue fluorescent band. As expected from previous observations (Tsuji, Chase and Harvey '55; Shimomura et al., '57), luminescence activity was associated with the yellow yellow fluorescent band, of which 4 separate fractions, F1–F4, were collected and dried in vacuo. The third fraction from the front, F3, was richest in luciferin with a luminescence potency of 300,000 L.U./mg; portions of this fraction were used for spectroscopic studies and for ascending paper chromatography. The quantity of material available seemed too small to justify an attempt at crystallization.

Absorption spectrum, and changes on standing

The absorption spectrum of fraction F3 just after dissolving in methanol is illustrated in figure 1, curve A. It is very similar to that of the methanolic solution of crystalline luciferin reported by Shimomura et al., ('57, fig. 2, p. 930), differing only in having a shoulder rather than distinct maximum at 315 mμ, (a difference possibly due to a difference in pH; cf. discussion of fig. 3), and in having about a 50% larger ratio between optical density at 270 and the O.D. at 435 mμ, i.e. a ratio of approximately 3:1 instead of 2:1, indicative of some impurities that absorb in the ultraviolet.

The changes in absorption on standing aerobically at room temperature (fig. 1, curves B and C) are also similar to those of crystalized luciferin referred to above, and the isosbestic point at 390 mμ shows that no intermediary product accumulates. The changes in optical density of the 435 mμ maximum from the initial value, after two days, and after 8 days, closely parallel the changes in luminescence potency.

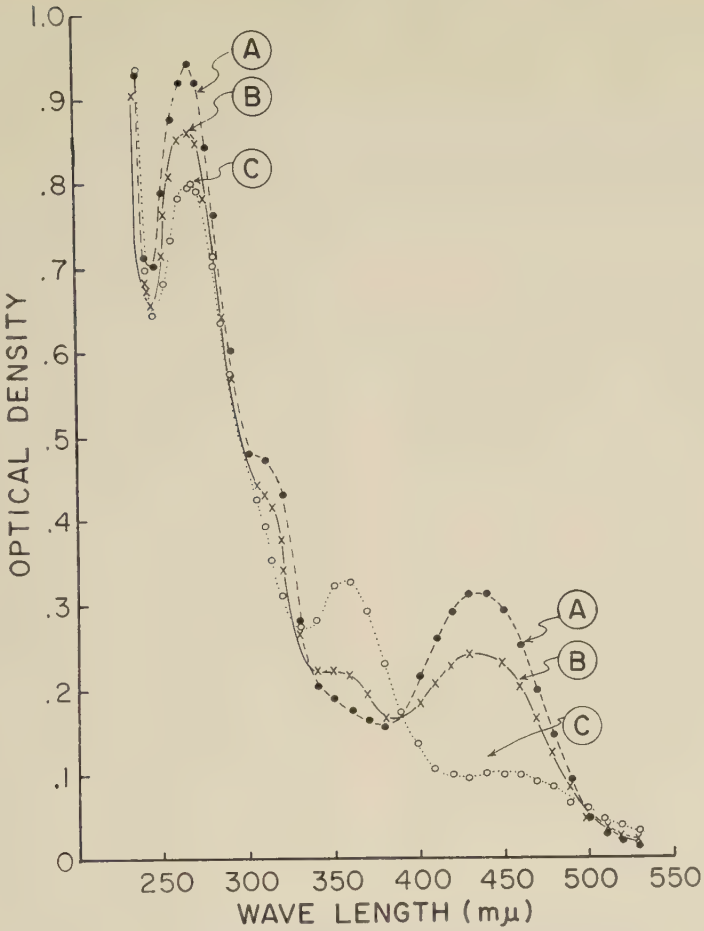

Fig. 1 Changes in the absorption spectrum of luciferin fraction F3, in a concentration of 0.108 mg/ml methanol, at room temperature in the presence of air, as described in the text. Curve A, just after dissolving; curve B, after two days; curve C, after 8 days.

us, the ratio of O.D.'s at these three cessive times is 0.320:0.245:0.099, or 0:0.78:0.31, while the ratio of lumi- cence potency at the corresponding es was 300,000:234,000:45,000, or 0:0.78:0.15. These data leave little bt that the absorption maximum at 5 mμ is characteristic of the substance, , luciferin, that is active in light emis- n with luciferase.

Quantitative determinations of the total t potency of solutions of crystalline iferin obtained in the present study e yielded values of 3.7 to 4.6 million . per mg, varying with the luciferase paration employed. Taking 4×10^6 ./mg as a mean value, the 300,000 L.U./mg in fraction F3 would indicate the presence of 7.5% luciferin, or 0.0081 mg/cm³ in the specimen involved. The initial optical density of 0.32 at 435 mμ would indicate 0.0171 mg/cm³, but when 85% of the activity has disappeared on standing, the O.D. remains 0.1, whereas the decomposition products of pure luciferin have practically no absorption at this wave length. Assuming that impurities absorbing at 435 mμ have an O.D. of 0.085, the amount of luciferin represented by the absorption at this point would be 0.0125 mg/cm³, or 11.5% luciferin in the specimen. Considering the various sources of error, these estimates of 7.5% luciferin, on the basis of light emitting potency, and

11.5% luciferin, on the basis of O.D. at 435 mμ, are in reasonably good agreement. Thus it should be possible to estimate with some assurance the actual quantity of pure luciferin in different lots of raw material through determinations of the number of light units emitted per mg, when tested with luciferase under appropriate conditions.

Paper chromatography

The results of ascending chromatography on Whatman paper no. 1 varied with the developing solvent, very likely due in part to the circumstance that facilities for complete anaerobiosis, essential for preventing oxidative decomposition of luciferin, were not available for the papers at that time, so developing was carried out at 2°C overnight. With Tsuji's solvent, a trace of orange-fluorescent substance at the front and a trace of blue fluorescent substance behind the broad, yellow colored, yellow fluorescent, active band was observed. With butanol two active bands, evidently corresponding to the alpha and beta luciferin of Mason and Davis ('52), were found at the origin and at an intermediate point from the front respectively; the latter band exhibited a blue fluorescence in UV, and though quite active in light emission with luciferase, was not in sufficient amount to exhibit color by ordinary light. With acetone most of the color, fluorescence and activity remained at the origin, but a trace of yellow color, yellowish fluorescence and luminescence activity with luciferase accurred at the front.

Chromatographic evidence for the existence of more than one species of luciferin molecules active in light emission with luciferase, such as the results of Mason and Davis ('52) and the results described above, is not fully convincing. Such evidence can be reasonably interpreted in terms of the influence of impurities, on the chromatographic behavior of one kind of luciferin. In unpublished experiments by Shimomura and associates, anaerobic paper chromatography of crystallized luciferin from dried organisms has revealed only a single yellow colored, yellow fluorescent, active band with Tsuji's solvent, and attempts to separate two distinctly different, pure luciferins by using other solvents or combinations of solve have been unsuccessful. Moreover, results of the present study indicate t the crystalline luciferin previously tained from dried organisms is the sa as that obtained more directly from fres collected organisms, quickly frozen by ice and extracted at quite low tempe tures, as described in the following pa graphs.

Cold methanol extracts

Experiments with extracts from Shi oda revealed that luciferin remained solution in methanol at temperatures low −20°C, whereas considerable amou of colorless impurities came out and co be removed by refrigerated centrifugati In more recent experiments, live *Cypridi* collected at Tateyama were placed eith in methyl alcohol pre-cooled to abc −50°C in a polyethylene bottle, or w put into a thin polyethylene bag and th into a thermos jar containing dry ice. T latter method proved more convenient, a qualitative observations indicated that t organisms could be kept at the temperatu of dry ice virtually indefinitely withc perceptible loss of luminescence activ when thawed. By completing the init extraction of luciferin without allowi the temperature ever to rise above −20° the extracts in effect were prepared fro live organisms, and contained minim amounts of fat and other impurities.

The yield of luciferin was augmente by several repeated extractions with tw or three volumes of methanol per volum of organisms at −20 to −30°C in a va uum flask with the air exhausted an with vigorous shaking. The cold solutio was removed by rapid filtration throug paper in a Buchner, with suction. A goc yield could be obtained with a muc smaller volume of cold methanol by h mogenizing the organisms in a blendor an filtering the brei with the aid of hyflosupe cel and washing with cold methanol. B this method it was more difficult to main tain the temperature below −20°C unt filtration had been completed, and dry ic usually had to be added to the brei.

The initial fractionation was thus ac complished by the differential solubility c luciferin in methanol at low temperature

e filtrate was always bright yellow in or, with strong yellow or greenish-yel- v fluorescence in U.V. It was acidified adding concentrated HCl to make the ution about 0.1 N acid, in order to con- rt the luciferin to a more stable state, d the methyl alcohol removed by vac- m distillation in a water bath at about °C, with a dry ice-acetone trap in the cuum line, and a capillary stem sup- ed with pure H_2 at reduced pressure, in e flask to prevent bumping. The residual ueous solution was extracted several nes with benzene, whether or not colored bstances went into the benzene. In me instances, reddish material contain- g active luciferin remained at the inter- ase. Large, orange colored crystals of t fully pure luciferin were obtained from tanol solutions of such material by eans of the usual further procedure de- ribed below.

Most of the luciferin in the acid, aque- s solution could be partitioned into bu- nol in a separatory funnel with an argon mosphere. Saturated $NaHCO_3$ solution as added to neutralize the acid in the tanol layer, and the water layer was scarded. The butanol layer was concen- ated to a small volume by vacuum dis- lation, and placed on a column of Brock- an's standarized alumina in an atmos- ere of hydrogen. The column assembly diagrammed in figure 2.

On washing with butanol, the column sually showed three bands, in addition some brownish, amorphous material, ssibly denatured protein and inorganic alts, which remained on top: (1) a owly moving, yellow colored, yellow flu- escent substance identified with active ciferin, (2) a pink to red material, in- tive with luciferase moving more slowly an luciferin, and (3) a colorless, blue uorescent material which stayed on the olumn. Mixtures of butanol and meth- nol, in proportions of 4:1 to 2:1 by vol- me, were used to elute the luciferin when moved too slowly in butanol alone.

The eluate was dried by vacuum distil- tion in a water bath at 50 to 70°C, leav- ng a yellowish film, sometimes with small mounts of white material. The film dis- olved readily in a few tenths of one ml f cold methanol. To the solution in a 15

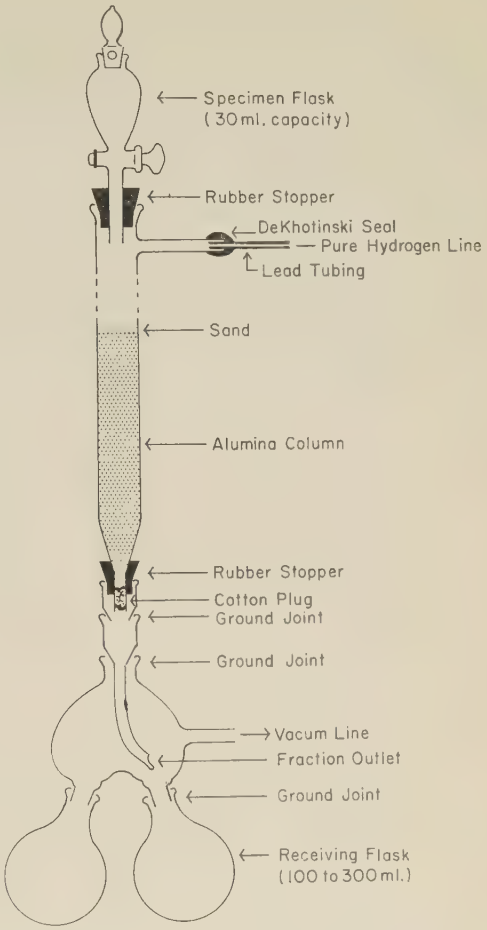

Fig. 2 Diagram of the apparatus used for anaerobic chromatography of luciferin on alumina. The column length was adjusted, from about 4 to 12 cm, in accordance with the estimated quantity of luciferin in the specimen involved. The whole assembly was evacuated and filled with hydrogen three successive times before introducing the specimen. The fraction outlet could be rotated into position over any one of three receiving flasks without exposing the eluate to oxygen.

ml centrifuge tube, three to 5 drops of distilled water were added, and a few small drops of 12 N HCl. In some instances, crystallization began almost at once. As a rule the centrifuge tube was placed in a vacuum desiccator over P_2O_5, with a beaker of KOH pellets on the porcelain support, the air was exhausted to 12 cm Hg, followed by filling with pure hydrogen and exhausting to this pressure

three times, and the desiccator placed in a refrigerator overnight. The crystals were then filtered off on a small sintered glass filter and dried in a vacuum desiccator. Figure 3 illustrates the absorption spectrum of the product, obtained in this manner, in comparison to that which was first reported (Shimomura et al., '57). The slight differences in the two curves are very likely due to slight differences in pH to which the absorption spectrum is particularly sensitive (Shimomura, '60).

By the above procedure, crystalline luciferin could be obtained in one day from about 0.5 kg wet weight of organisms had been kept in dry ice. Purity and e of crystallization were favored by sp in carrying out the procedure. The yi however, was favored by starting wit larger amount of raw material, and 2 wet weight, proved to be about optir though more than one day was required purification. The partially purified l ferin, after partitioning into butanol, cc be kept satisfactorily, for several day necessary, in a small flask in a therr jar with dry ice.

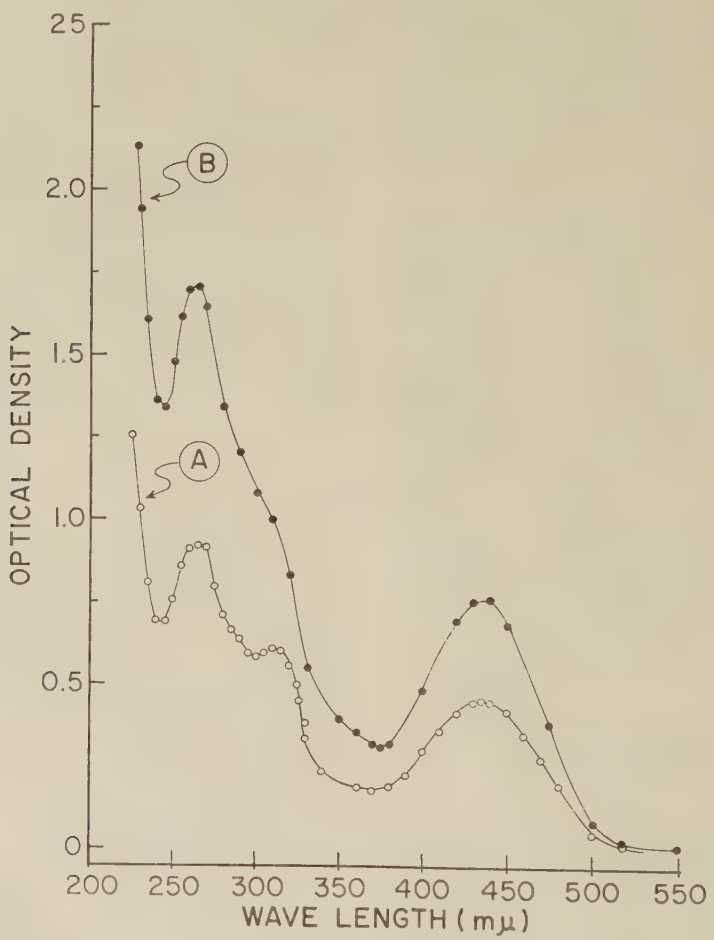

Fig. 3 Absorption spectrum of a methanolic solution of (A, open circles) crystalline luciferin 0.032 mg/cm³, from dried *Cypridina* (Shimomura, Goto and Hirata, '57, plotted from the original data) and (B, solid circles) of crystalline luciferin, 0.041 mg/cm³, obtained from cold methanol extracts of deep frozen, initially live *Cypridina*, as described in the text. Data for curve A were taken with a Beckman DU spectrophotometer, temperature 10°C. Data for curve B were taken on a Hitachi recording spectrophotometer, temperature 29°C.

The yield at best amounted to about mg crystalline luciferin from 2 kg wet ight of frozen organisms. Assuming a % to 80% yield, the living organisms uld contain an average of 0.0025 to 004% luciferin by weight. Since water nstitutes about 75% of the wet weight, e actual yield would correspond to 80 mg r kg of dried organisms, provided no s occurred during drying. The best ld, however, from organisms collected cold seasons and dried, has been about mg/kg dry weight, and heretofore it s been extremely difficult to obtain apeciable quantities of crystalline luciferin m summer *Cypridina*. It is reasonable conclude that the luciferin content of e, cold season organisms is at least an der of magnitude higher than that of mmer organisms. If so, the cold methol procedure should yield at best 400 mg crystalline luciferin per kg wet weight winter organisms. Again assuming a to 80% yield, the cold season, living ganisms would contain between 500 and 5 mg/kg, or between 0.050 and 0.0625% ciferin by weight. These figures are one two orders of magnitude lower than e value of 1% to 10% used by Harvey 3) in estimating that the minimum nount of luciferin needed for the proction of visible light in reacting with ciferase was one part of luciferin in m 4 to 40 billion parts of water; an timate of one part of luciferin in from ^{10} to 10^{11} parts of water appears to be ore nearly correct.

The light-emitting potency of the first ystalline luciferin (Shimomura et al., 7) was estimated as being almost 40,000 nes that of the starting material. Under vorable conditions of assay, pure luciin would be expected to have a constant tency, whereas the actual content in the arting material is subject to wide variaons, as discussed above. The increase in tency on purification, therefore, might expected to range from perhaps 5,000 100,000 times depending on the activity the starting material. Moreover, since e "potency" is subject to variation with e efficiency of light production, in both ude and fully purified systems, through e influence of numerous factors, such as H, temperature, specific ions, etc., as discussed in detail elsewhere (Johnson, Eyring and Polissar, '54), the above range under appropriate conditions might well be even greater.

SUMMARY

A simplified method is described for purification of the substrate, luciferin, in the luminescent enzyme, luciferase, system of *Cypridina* by extraction of initially living, whole organisms with methanol.

Extraction in vacuo with hot (65°C) methanol yielded a product containing about 10% luciferin, by distilling off the methanol in vacuo, de-fatting the aqueous residue with benzene, partitioning into butanol, and chromatographing on a cellulose column with Tsuji's alcohol-acetate-water solvent.

Quantitative data revealed that the decrease in light-emitting potency of the product, on standing aerobically in methanolic solution two days at room temperature, was the same as the decrease in optical density at 435 mμ, viz., 22%. After 8 days, luminescence potency had decreased by 85%, with a 69% decrease in O.D. at this wavelength.

Extraction with cold (−20°C) methanol, using summer organisms kept at dry ice temperature since shortly after collecting, yielded crystalline luciferin in an amount of approximately 20 mg/kg wet weight, by the same purification procedure except that the chromatography was on a column of alumina with butanol as the solvent. The absorption spectrum of the product was essentially identical with that of the luciferin first crystallized from dried organisms.

The luciferin content of living Cypridina collected in summer is estimated as averaging 0.0025 to 0.004% by weight, and perhaps more than 10 times this amount in organisms collected in cold seasons.

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